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Advances of diagnostic and mechanistic studies of γ-glutamyl transpeptidase in hepatocellular carcinoma

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Summary **Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second major cause of cancerous deaths in the world, accounting for 80-90% of all cases of liver cancer with an assessed global incidence of 782,000 new cases and approximate 746,000 deaths in 2012. Preoperative laboratory data (des-γ carboxyprothrombin (DCP), α-fetoprotein (AFP), Indocyanine green retention 15 min (ICG-R15), and γ-glutamyl transferase (GGT)) should be completely assessed before deciding a treatment and predicting prognosis in order to improve the prognosis for patients with HCC. A few recent studies have suggested GGT as an independent prognostic indicator in cases with HCC. And the data of our and other research teams revealed that combination of GGT and ICG-R15 or other factors may improve the efficiency of GGT as a prognostic predictor. In addition of clinical studies, a few mechanistic studies had been performed and GGT was suggested to promote tumor progression and poor prognosis through inducing DNA damage and genome instability, releasing reactive oxygen species to activating invasion-related signaling pathway, blocking chemotherapy, regulating microRNAs, and managing CpG island methylation. Although there were a few mechanistic studies, further and accurate researches were still in need.**

Keywords: γ-Glutamyl transferase (GGT), indocyanine green retention 15 min (ICG-R15), prognosis, risk factor, hepatocellular carcinoma (HCC)

1. Introduction

Hepatocellular carcinoma (HCC) is the second chief culprit of cancer deaths worldwide. HCC caused a global incidence of 782,000 new sufferers and almost 746,000 deaths in 2012 (*1*). At present, hepatic resection is considered as the first treatment option for early stage HCC. Although improved diagnostic methods, surgical techniques, and perioperative period management have lead to better results (*2-5*), the striking rate of recurrence after hepatectomy is still a barrier that deteriorated patient prognosis, with a cumulative rate of 50-60% at 3 years and 60-80% at 5 years (*6,7*). As a result, there is an urgent need for surgeons to find out how to predict prognosis and take interventional measures as early as possible.

Up to now, certain risk factors of the prognosis of HCC have been studied, and some factors such as microvascular invasion (MVI), poor differentiation, and tumor size have been validated as important risk factors impairing prognosis after hepatectomy (*8*). Lately, a great number of studies on various subgroups of cases, such as patients with hepatitis B virus (HBV)-related HCC, hepatitis C virus (HCV)-related HCC, noncirrhotic HCC, non-alcoholic fatty liver disease-related HCC, or multinodular tumors, have investigated risk factors which predict prognosis of sufferers with HCC (*9,10*). And for these years, a series of biochemistry factors, such as α-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP), indocyanine green retention 15 min (ICG-R15),

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Table 1. Factors related to prognosis for patients with HCC

Examinations	Indicators	Applications
Laboratory data	AFP	Tumor marker in liver cancer
	DCP	Tumor marker in HCC
	GGT	Diagnostic marker for liver disease
	ICG-R15	Biomarker for liver reserve function
	GP73	Diagnostic marker for liver disease
	ALT	Biomarker for inflammation and liver injury
	HBsAg	Biomarker for HBV infection
	Platelet count	Diagnostic marker for liver disease
	COMP	Biomarker for liver fibrosis and early HCC
	AGE	Biomarker for cancer growth, and metastasis
Imaging data	Tumor size	
	Tumor number	
	Vascular invasion	
Pathological data	Tumor differentiation	
	Microvascular invasion	
	Intrahepatic metastasis	

AFP: α-fetoprotein; DCP: Des-γ-carboxyprothrombin; GGT: γ-glutamyl transferase; ICG-R15: Indocyanine green retention 15 min; GP73: Golgi protein 73; ALT: Alanine aminotransferase; HBsAg: Surface antigen of the hepatitis B virus; COMP: Cartilage oligomeric matrix protein; AGE: Advanced glycation end products.

and γ-glutamyl transpeptidase (GGT), had been studied and utilized as risk predictor for tumor progression and prognosis. Among them, GGT attracted more attention for its advantage of predicting the postoperatively prognosis.

GGT is an enzyme that transfers γ-glutamyl functional groups (*11*). It exists in the cell membranes of many tissues and involved in glutathione metabolism by transferring the glutamyl moiety to a variety of acceptor molecules including water, certain L-amino acids, and peptides, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress. There are increasing amount of researches suggesting that GGT may play an important role of predicting prognosis for patients with HCC.

2. GGT as a predictive biomarker in clinical investigations

In Table 1, laboratory data, imaging data, and pathological data have identified some indicators as risk factors of prognosis for sufferers with HCC (*12-15*). Microvascular invasion (MVI), tumor size, and tumor number indicated in imaging data are considered as risk factors for prognosis. And imaging studies have been given weight before deciding a treatment and predicting the prognosis for patients with HCC. However, as some research have revealed, a tumor may recur in approximately 60.0% of sufferers with a single tumor smaller than 2.0 cm (*16*). As a result, more methods to predict prognosis risk factors are urgently needed besides imaging data. Pathological data cannot validate pathologic changes prior to operation. In contrast, laboratory testing of AFP, DCP, ICG-R15, and GGT can be performed before surgery. Therefore, these indicators should be considered as a way to select a treatment and predict survival and recurrence for patients with HCC.

Patients with positive laboratory data for AFP, DCP, ICG-R15, and GGT have a higher risk of poor prognosis (*17-19*). These sufferers should be administrated with more effective treatments including anatomical hepatectomy, liver transplantation, preoperative and postoperative transcatheter arterial chemoembolization (TACE), and timely follow-up. Laboratory data for DCP and AFP are correlated to malignant conditions such as MVI and metastasis. ICG-R15 is suggested to be correlated to liver function (*17,20*). Lately, GGT has been validated as an independent prognostic risk indicator for patients with HCC (*18,21*).

GGT is a critical enzyme which catalyzes the hydrolysis of glutathione and the transfer of γ-glutamyl residues, and GGT has been widely utilized as a biomarker for some tumors, such as lung cancer and ovarian cancer. GGT was researched and employed as a liver function indicator in the 1960s to 1970s (*22*). An increasing level of GGT can be detected in patients with hepatitis, steatosis, cirrhosis, or HCC at various stage (*23,24*). Up to now, a great number of clinical studies have reported a high level of abnormal GGT in sufferers with primary or secondary HCC. According to a study by Tsutsumi *et al*., detection of mRNA expression of GGT could be a useful method for diagnosis of HCC at the early stage because GGT mRNA may change from type-I to type-II during the progress of HCC (*25*). But GGT is found to be abnormal in most cases with different liver diseases, and a large number of various diseases and conditions (such as pancreatitis, obesity, and alcohol abuse) can also lead to high expression levels of GGT (*25-27*). Therefore, GGT was not regarded as a useful tumor indicator for detecting HCC for a long time. GGT was utilized as a diagnostic tumor biomarker for liver disease with a high sensitivity of 83-100%, but it only has a low specificity of 32% (*10*). Therefore, long since GGT was not utilized as an

Table 2. Investigation of GGT as a prognostic factor based on different subgroups of patients with HCC

Authors	Patients	Treatment	Results
Song *et al*. (*36*), 2015	384 cases	Hepatectomy	GGT > 50 U/L is significantly associated with poor RFS
Wang *et al*. (*37*), 2014	288 cases	Hepatectomy	GGT > 55 U/L is significantly associated with poor RFS
Zhao *et al*. (*34*), 2013	266 cases with multi-nodular HCC	Hepatectomy	GGT > 130 U/L was a preoperative predictor for microvascular invasion
Chen *et al*. (*38*), 2014	154 cases	TACE	GGT > 85 U/L is significantly associated with poor RFS
Hung *et al*. (*39*), 2013	150 cases	TACE and chemotherapy	GGT > 100 U/L is significantly associated with poor RFS
Nishigawa *et al*. (*40*), 2013	74 cases with HBV-related HCC	Entecavir	GGT > 50 U/L is found to be significant prognostic factors linked to RFS
Nishigawa *et al*. (*41*), 2014	368 cases with solitary HCC	Radiofrequency ablation	GGT > 80 U/L is significantly associated with poor RFS

effective risk factor for the detection of liver disease. Nevertheless, GGT has important clinical significance as a predictive biomarker of prognosis. This finding was reported by researches based on different subgroups of cases published over the past five years. In the light of a study by Sheen *et al*., patients with HCC with type-II GGT mRNA had poorer prognosis, such as worse results, earlier recurrence, and higher death rates (*28*). A few studies of cases with HCC receiving hepatectomy have suggested a relationship between increasing levels of GGT and decreasing level of survival rate for patients with HBV-related HCC, Child-Pugh A liver function, or multi-nodular tumors (*29*). Moreover, a few studies have showed the predictive value of GGT in cases with unresectable HCC who received TACE or chemotherapy (*30-35*). In a clinical study of our research team, patients operating characteristic curves of 384 cases with single primary HCC who received hepatectomy were charted to validate the topgallant cutoff value of GGT was 50 U/L for recurrence-free survival (RFS) and 100 U/L for survival. After above analysis, GGT > 50 U/L was considered as a preoperative independent predictor impairing 1-, 3-, and 5-years RFS; GGT >100 U/L was considered as a independent predictor impairing 1-, 3-, and 5-years survival before operation. These results further validate the function of GGT as a preoperatively independent predictor correlated with tumor recurrence and overall survival in cases with HCC.

In Table 2, sufferers with high levels of GGT were apt to commit early recurrence and lower overall survival rate, including sufferers with multi-nodular HCC, HBV-related HCC, and those who received TACE, radiofrequency ablation, or entecavir. Hepatectomy, ultrasonography, CT and MR imaging and a timely follow-up are advised for these patients

Table 3. GGT combined with other risk factors

Authors	Patients	Factors
Song *et al*. (*36*), 2015	384 cases	GGT + ICG-R15
Norman *et al*. (*42*), 2015	187 cases	GGT + COMP
Cho *et al*. (*43*), 2014	337 cases	GGT + MPV
Kan *et al*. (*44*), 2014	90 cases	GGT + AGE
Hou *et al*. (*45*), 2013	79 cases	GGT + AFP + GP73

GGT: γ-glutamyl transferase; ICG-R15: Indocyanine green retention 15 min; COMP: Cartilage oligomeric matrix protein; MPV: Mean platelet volume; AGE: Advanced glycation end products; AFP: α-fetoprotein; GP73: Golgi protein 73.

(*29,36-40*). The combination of GGT and other indicators, such as tumor size, tumor number, MVI, or laboratory data for AFP and DCP, should be paid more attention when deciding a treatment and predicting the curative results for patients with HCC (*41*).

As shown in Table 3, recently, besides AFP and DCP there were more laboratory indicators which were thought highly of prognostic prediction and were combined with GGT to predict the recurrence. Norman *et al*. reported that combination of cartilage oligomeric matrix protein (COMP) > 15 U/L and GGT > 50 U/L was associated with cirrhosis and poor prognosis for patients with HCC (*42*). In 2014, Cho *et al*. published their research results that combination of mean platelet volume (MPV) and GGT was considered as malignant indicator (*43*). And in the same year, research result from Kan *et al*. suggested that advanced glycation end products combined with GGT would be indicators for non-B or non-C HCC (*44*). In 2013, Hou *et al*. reported that the combination of α-fetoprotein (AFP), Golgi protein 73 (GP73), and GGT might serve as a potential predictive method for HCC (*45*). In a clinical study of our team, GGT and ICG-R15 were focused on as

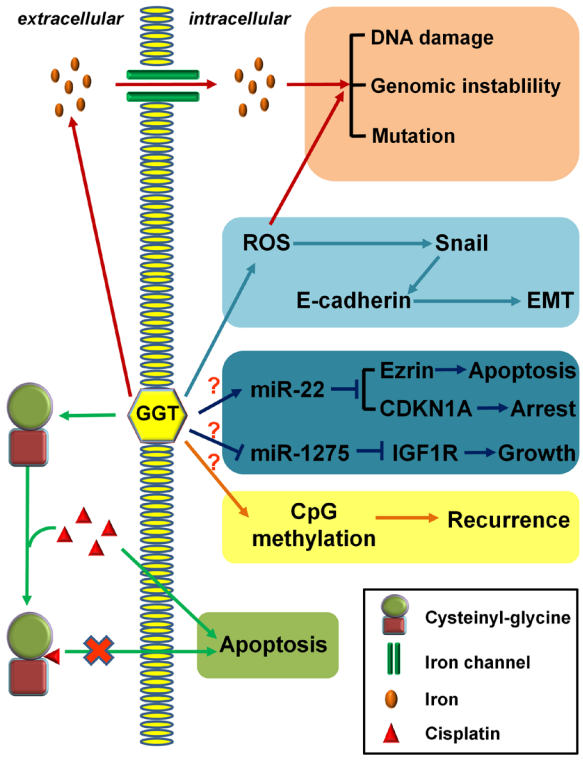

Figure 1. The molecular mechanisms of GGT in HCC.

predictors of prognosis in patients with single primary HCC who received hepatectomy (*36*). GGT > 100 U/L was considered as a preoperative independent predictor correlated with survival, and GGT > 50 U/L + ICG-R15 > 10% were considered as preoperative independent predictor correlated with tumor recurrence. Sufferers with GGT > 50 U/L + ICG-R15 > 10% commonly had a worse 1-, 3-, and 5-years RFS, and this was also true in cases with a tumor < 5 cm in size. These results indicate that combination of high levels of GGT and ICG-R15 should be paid more attention as a preoperative indicator correlated with prognosis for patients with single primary HCC receiving hepatectomy.

3. GGT as a functional macromolecule in mechanistic studies

The reason why GGT is significantly correlated to high level of recurrence and low level of survival has not yet to be illustrated. As shown in Figure 1, there are five possible mechanisms: *i*) GGT may be correlated to poor prognosis *via* leading to DNA damage and subsequent oncogenesis; *ii*) GGT may be correlated to the degree of malignant outcomings, such as MVI, metastasis, and epithelial-mesenchymal transition (EMT) through promoting certain signaling pathways; *iii*) GGT may be correlated to worse chemotherapeutic results by blocking the permeation of chemotherapy medicine into tumor cell; *iv*) GGT may be correlated to recurrence by regulating certain nucleic acid molecule to promote tumor growth and survival; *v*) GGT may be correlated to drug resistance and recurrence of HCC through leading to CpG island methylation in certain regains in genome.

An increasing number of researches have clarified mechanisms of GGT over the recent years. In a study, it was suggested that GGT lead to DNA damage, genomic instability, and oncogenesis-related mutations by promoting the uptake of iron (*46*), and the role of iron playing in carcinogenesis was already reported by Weinberg *et al*. (*47*). This mechanism is suggested to cause the death of normal liver cells or the destroying of normal liver function. The pro-oxidant function of GGT has been revealed and the subsequent product reactive oxygen species (ROS) may activate some intra- and extracellular molecular signaling pathways (*48*). Lately, ROS were suggested to induce EMT through the Snail/E-cadherin signaling pathway (*49*) and to promote inflammation and invasion by the NF-κB signaling pathway (*50,51*). Another research of U937 lymphoma cells discovered that GGT may play a role in anti-apoptosis (*52*). A research team has revealed that cysteinyl-glycine, which is catalyzed by GGT, is able to combine with cisplatin to form complexes which are not easily transported through the cell membrane (*53,54*).

In a study, it was revealed that increasing level of GGT was significantly related with changes of expression level of miR-22 and miR-1275 (*55*). In other researches, the abnormal expression levels of these two microRNAs were testified to promote anti-apoptosis and tumor growth (*56-58*). A recent study suggested that the up-regulated level of GGT was associated with the CpG island methylation in certain regains in genome, and CpG island methylation had already been reported to be correlated to tumor progression in various cancers (*59*). These mechanisms are considered to explain the progression and poor prognosis of HCC. Although the significance of molecular mechanisms of GGT to worse liver function, progression, and prognosis of HCC is indicated, these molecular mechanisms should be illustrated in further studies. From these studies it is not difficult for us to find that GGT may play a significant role in tumor progression and poor prognosis through various signaling pathways and mechanisms, thus GGT may become a novel target for tumor treatment in addition of as a predictor.

4. Conclusions

In conclusion, preoperative laboratory data (DCP, AFP, ICG-R15, and GGT) should be completely assessed before deciding a treatment and predicting prognosis in order to improve the prognosis for patients with HCC. A few recent studies have suggested GGT as an independent prognostic indicator in cases with HCC. In study of our research team, it was suggested that the preoperative role of GGT > 50 U/L and ICG-R15

> 10% as independent prognostic indicator for tumor recurrence in cases with single primary HCC who received hepatectomy. Patients with up-regulated levels of GGT and ICG-R15 had a worse 1-, 3-, and 5-years RFS. Therefore, combination of high levels of GGT and ICG-R15 could be useful for assessing prognosis postoperatively. In addition, some novel combination methods also may improve the prediction of prognosis of patients with HCC. Although a few molecular mechanisms of GGT were reported and these findings shed light on future functional study of GGT, the further and accurate mechanistic analysis was still in need.

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Original Article

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Design, docking analysis, identification, and synthesis of novel 3-(((substituted phenyl) amino)methyl)-2-methylquinazolin-4(3*H*)-one compounds to fight tuberculosis

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Summary **In this study, a series of novel scaffold-based 3-(((substituted phenyl)amino)methyl)-2-methylquinazolin-4(3*H*)-one compounds, 3a-3r, was synthesized, characterized, and screened for its *in vitro* activity against the H37Ra strain of *Mycobacterium tuberculosis*. A number of analogs were found to have highly potent anti-tuberculosis activity. Compound 3m in particular had potent activity equal to that of the standard anti-tuberculosis drug rifampicin. New leads can be generated with the model developed in this study and this model will be optimized with the eventual goal of preparing new anti-tuberculosis agents.**

Keywords: Quinazoline, amines, antimicrobial activity, antituberculosis activity

1. Introduction

Tuberculosis (TB) is one of the most common infectious diseases. Caused by *Mycobacterium tuberculosis*, TB kills two million people every year and it continues to be a major cause of morbidity and mortality all over the world. About one-third of the world's population is currently infected with TB (*1*). If the present trend continues, tuberculosis is likely to claim more than 30 million lives within the next decade. Five percent of all TB cases are now estimated to be multi-drug-resistant TB. This form of TB is resistant to the first-line drugs streptomycin, isoniazid, ethambutol, pyrazinamide, and rifampicin and to at least one of the injectable second-line drugs, contributing to the resurgence of the disease (*2,3*). The overall incidence of TB in HIV-positive patients is 50 times that of the rate for HIV-negative individuals (*4-6*). Hence, there is an urgent need to discover and develop new anti-TB agents that target novel biochemical pathways and to treat drug-resistant forms of the disease. Quinazolin-4(3*H*)-one and related quinazolines are classes of fused heterocycles that are of considerable interest because of the diverse range of their biological properties. Quinazolin-4(3*H*)-one with a substituted group at position 3 has diverse therapeutic activities including antibacterial (*7*), antifungal (*8*), antimalarial (*9*), anticancer (*10*), antiinflammatory (*11*), anticonvulsant (*12*), and analgesic (*13*) activity. Quinazoline and its derivatives with a different pharmacophore group each have different modes of action in the treatment of TB. In addition, a survey of the literature revealed that quinazoline and quinazolinone systems have yielded numerous derivatives with anti-TB activity (*14*). Attention has been drawn to the earlier finding that some quinazoline-based compounds are lipophilic since interactions between ligands and target molecules and permeability are important parameters that determine drug action (*15*). In the search for novel scaffold-based antimycobacterial agents, a library of quinazolin-4(3*H*)-one derivatives that was constructed by this Laboratory was screened for action against the drug-susceptible H_{37}Ra strain of *M. tuberculosis*. The action of these compounds was compared to that of the standard anti-TB drugs rifampicin and isoniazid (INH).

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2. Materials and Methods

2.1. *Materials*

Synthetic starting material, reagents, and solvents were of analytical grade or of the highest quality commercially available. Chemicals were purchased from Aldrich Chemical Co., Merck Chemical Co., and Biotium, Inc. (Ambala, Haryana, India) and were dried whenever necessary. Melting points were determined in open capillary tubes and are uncorrected. Infrared (IR) spectra were recorded with KBr pellets (ABB Bomem FT-IR spectrometer MB 104 from ABB Limited, Bangaluru, India). Proton nuclear magnetic resonance (^{1}H NMR) spectra (Bruker NMR spectrometer, Punjab University, Chandigrah, India) were recorded with tetramethylsilane as an internal reference. Mass spectral data were recorded with a quadrupole mass spectrometer (Shimadzu GC MS QP 5000, Punjab University, Chandigrah, India) and microanalyses were performed using a *vario* EL V300 *elemental* analyzer (Analysensysteme GmbH, Chennai, India). The purity of the compounds was determined with thin-layer chromatography on aluminium plates pre-coated with SiO_2 gel (HF_{254}, 200 mesh) (E. Merck, Ambala, Haryana, India). IR, ^{1}H-NMR, mass spectral data, and elemental analyses were consistent with the proposed structures.

2.2. *General procedures*

A mixture of anthranilic acid (1.37 g, 0.01 mol) and acetic anhydride (10.2 mL, 0.1 mol) was refluxed over a gentle flame for 1 h. Excess acetic anhydride was distilled off under reduced pressure and the residue was dissolved in petroleum ether. The residue was left to stand for 1 h. 2-methyl-4*H*-benzo[1,3]oxazin-4-one **1** was obtained as a light brown solid, and this solid was filtered and dried. A mixture of 2-methyl-4*H*-benzo[1,3]oxazin-4-one **1** (0.01 mol) and ammonium acetate (0.01 mol) was combined in an oil bath for 2 h. The mixture was poured into an ice/water mixture and stirred. The precipitate that separated out was filtered, washed, dried, and then recrystallized from ethanol, yielding 2-methylquinazolin-4(3*H*)-one **2** as beige crystals. The appropriate substituted aromatic amines (0.02 mol) were slowly added to a solution of **2** (0.01 mol) in glacial acetic acid (50 mL) containing 37% formalin (1 mL). The reaction mixture was refluxed in a water bath for 1-3 h. The reaction mixture was concentrated to approximately half its initial volume, and the resulting precipitate was recrystallized from ethanol to yield a pure form of 3-(((substitutedphenyl) amino)methyl)-2-methylquinazolin-4(3*H*)-one compounds **3a-3r**. Table 1 shows the physical data for the synthesized compounds.

2.3. *In silico study*

Data on the synthesized compounds that docked in the crystal structure of DNA gyrase were obtained from the Protein Data Bank (PDB entry code 1KIJ2) and analyzed using Auto Dock 4.2. All polar hydrogen atoms were added and partial charges were placed with the help of the GROMACS package. The energy of the molecule was minimized by keeping heavy atoms fixed at their initial crystal coordinates and the added hydrogen atoms were allowed to move. Minimization was performed under a vacuum. Electrostatic interactions were calculated using the cut-off method. Solvation parameters were added using the ADDSOL option in Auto Dock 4.2. Default values for atomic solvation parameters were used throughout the calculations. Grid maps of the protein were calculated using the program

Table 1. Physical data on 3-(((substituted phenyl)amino)methyl)-2-methylquinazolin-4(3*H*)-one 3a-3r

Compounds	R1	Mol. formula	% yield	M.p (°C)	R_f[a]
3a	*o*-F	$C_{16}H_{14}FN_3O$	70	245-247	0.56
3b	*m*-F		76	215-217	0.51
3c	*p*-F		81	212-214	0.66
3d	*o*-Br	$C_{16}H_{14}BrN_3O$	79	223-225	0.72
3e	*m*-Br		72	241-243	0.74
3f	*p*-Br		74	262-265	0.55
3g	*o*-Cl	$C_{16}H_{14}ClN_3O$	73	211-213	0.62
3h	*m*-Cl		70	210-212	0.74
3i	*p*-Cl		86	272-274	0.83
3j	*o*-NO_2	$C_{16}H_{14}N4O_3$	78	223-225	0.58
3k	*m*-NO_2		71	245-247	0.67
3l	*p*-NO_2		82	231-233	0.79
3m	*o*-CH_3	$C_{17}H_{17}N_3O$	71	246-247	0.69
3n	*m*-CH_3		70	245-248	0.68
3o	*p*-CH_3		85	237-239	0.59
3p	*o*-OCH_3	$C_{17}H_{17}N_3O_2$	72	222-224	0.56
3q	*m*-OCH_3		71	228-230	0.81
3r	*p*-OCH_3		87	245-247	0.63

[a]The solvent system used was ethylacetate/hexane/methanol (4:6:2 v/v).

AutoGrid. The ligands used for docking were drawn using MDL ISIS Draw 2.5 and saved as a 'mol' file that was imported into ACD/ChemSketch to obtain a file format compatible for use in ArgusLab. After 3D optimization, molecule data were stored as a PDB file. All possible flexible torsions of the ligands were defined by AUTOTORS. Docking simulations were done with Auto Dock 4.2 using a Lamarckian genetic algorithm. Standard docking procedures were used for rigid proteins and flexible ligands with torsion angles that were identified. A grid consisting of 60, 60, and 60 points in the x, y, and z directions was created with its center at the catalytic site of the protein. Default settings were used for all other parameters. All calculations were performed on PCs running Windows XP. The resulting structures were analyzed using Auto Dock Tools.

2.4. *MABA Assay protocol*

The prepared compounds were tested for their *in vitro* activity against the $H_{37}Ra$ strain of *M. tuberculosis*. An antimycobacterial bioassay was performed using the microplate Alamar Blue assay (MABA) (*16*). Briefly, representative colonies of the $H_{37}Ra$ strain from the Lowenstein-Jensen (LJ) slope were suspended in 1 mL of distilled water and the turbidity was adjusted to match McFarland tube No.1 (10^7 CFU/mL). This suspension was further diluted to 1:25 in 7H9 (Middlebrook 7H9 [Becton Dickinson] supplemented with 0.2% glycerol, 0.1% casitone, and 10% albumin-dextrose, pH 6.8) and used as the inoculum. One hundred µL of the bacterial suspension was added to each well of a micro titer plate together with the synthesized compounds **3a-3r** in Middlebrook 7H9 medium to reach a final volume of 200 µL. The final concentration of the test compounds, **3a-3r**, ranged from 31.25 µg/mL to 0.97 µg/mL. A growth control well and a sterile control well were also included on each plate. Plates were covered and sealed with Parafilm and incubated at 37°C. After incubation for about 7 days, 20 µL of Alamar Blue dye were added to the wells. The plates were re-incubated overnight. A color change from blue to pink indicated bacterial growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of a compound that inhibited visible bacterial growth of *M. tuberculosis* and the MIC ranged from 0.0047-0.0095 (µg/mL). The first line anti-TB drug INH served as the reference compound.

3. Results and Discussion

3.1. *Chemistry*

The series of heterocycles, 3-(((substituted phenyl) amino)methyl)-2-methylquinazolin-4(3*H*)-one **3a-3r**, was synthesized by the reaction of 2-methylquinazolin-4(3*H*)-one **2** with an appropriate solution of substituted aromatic amines as shown in the **Diagram**. The IR

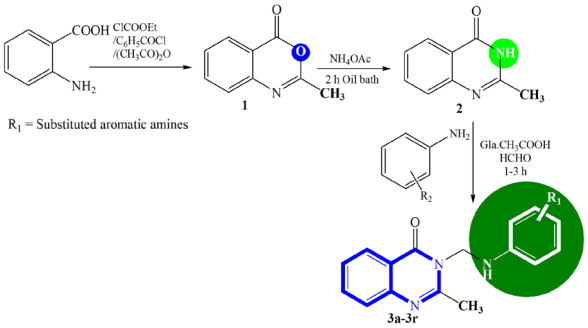

Diagram. Protocol for synthesis of the target compounds 3a-3r

spectrum had a strong stretching band at 3,229 cm^{-1} due to the secondary amino group and no C-O-C stretching peak in the range of 1,300-1,300cm^{-1}, confirming the formation of compound 2-methylquinazolin-4(3*H*)-one **2**. The IR spectra of the obtained compounds had stretching bands for C-F (1,009, 1,101, 1,031cm^{-1}), C-Br (642, 598, 627cm^{-1}), C-Cl (722, 714, 742cm^{-1}), and N-O (1,502, 1,542, 1,498 cm^{-1}), confirming the identity of compounds **3a-3l**. Compounds **2** and **3a-3r** were formed based on the NH stretching peaks in the range of 3,300-3,500 cm^{-1}. According to ^{1}H NMR spectra, the obtained compounds produced a single peak at 9.31, 8.48, 9.24, 8.94, 9.14, 8.94, 8.99, 9.48, 9.14, 9.11, 8.41, 9.44, 8.93, 8.41, 9.34, 8.95, 8.49, 9.12, and 8.71 ppm due to the NH group, confirming the formation of the compounds in question (**3a-3r**). The conversion of 2-methylquinazolin-4(3*H*)-one **2** was apparent from a single peak at δ 4.55, 4.27, 4.47, 4.25, 4.12, 4.12, 4.35, 4.47, 4.42, 4.51, 4.24, 4.51, 4.23, 4.57, 4.56, 4.47, and 4.51ppm due to the CH_2 proton, so ^{1}H NMR confirmed the formation of the compounds in question (**3a-3r**). In addition, mass spectra revealed the purity and molecular weight of those compounds. The above findings clearly indicated that the target compounds **3a-3r** were formed as indicated in the **Diagram** and had structures as were proposed.

3.2. *In silico screening*

Inhibition of DNA gyrase by 3-(((substituted phenyl) amino)methyl)-2-methyl quinazolin-4(3*H*)-one compounds **3a-3r** was indicated since they docked with the active site of DNA gyrase. Inhibition was tested experimentally, and the obtained Ki values correlate with that effect. The different scoring functions provide multiple approaches to evaluate ligand-receptor interactions and differing scores are expected to better aid in prioritization. Compounds **3m**, **3e**, **3b**, **3n**, **3q**, **3i**, and **3f** had the least free energy of binding. The free energy of binding and residues heavily interacting with compounds are shown in Table 2 and Figure 1. As an example, compounds **3m** (*o*-CH_3), **3e** (*m*-Br), and **3b** (*m*-F) had a free energy of binding of -1.19 kcal/mol and the ligand **3m** was found within the active site

Table 2. *In silico* and *in vitro* data on 3-(((substituted phenyl)amino)methyl)-2-methylquinazolin-4(3*H*)-one 3a-3r

Compounds	Substitution	ΔG	Ki	MIC (µg/mL)
3a	*o*-F	-4.4	590	31.25
3b	*m*-F	-5.2	153.2	1.95
3c	*p*-F	-3.95	1.26	1.95
3d	*o*-Br	-4.75	330.8	15.62
3e	*m*-Br	-5.23	147.2	1.95
3f	*p*-Br	-5.0	215.7	7.81
3g	*o*-Cl	-4.91	250.38	15.62
3h	*m*-Cl	-4.76	321.95	15.62
3i	*p*-Cl	-5.16	165.46	7.81
3j	*o*-NO_2	-4.55	458.83	31.25
3k	*m*-NO_2	-3.45	2.95	Nil
3l	*p*-NO_2	-3.92	1.34	Nil
3m	*o*-CH_3	-5.38	114.23	1.95
3n	*m*-CH_3	-5.2	153.41	7.81
3o	*p*-CH_3	-4.63	406.25	15.62
3p	*o*-OCH_3	-4.61	418.17	15.62
3q	*m*-OCH_3	-5.17	163.18	7.81
3r	*p*-OCH_3	-4.3	705.6	31.25
Rifampicin	-	-	-	0.0095

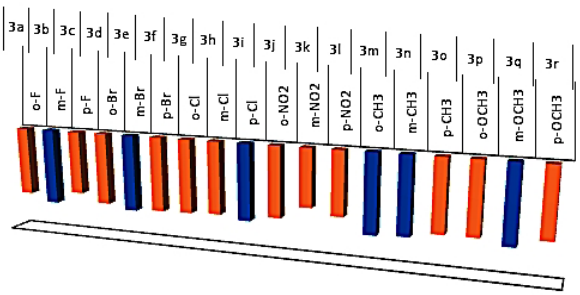

Figure 1. Graph of the binding energy (ΔG) of 3-(((substituted phenyl)amino)methyl)-2-methyl quinazolin-4(3*H*)-one 3a-3r.

residues ASN 715, ALA 765, LEU 712, LEU 766, VAL 763, TYR 237, and GLY 764 (Figure 2).

3.3. *Screening of in vitro anti-TB activity*

MABA was used to assay all 18 of the 3-(((substituted phenyl)amino)methyl)-2-methylquinazolin-4(3*H*)-one analogs, **3a-3r**, for their activity against *M. tuberculosis* strains at six different concentrations: 0.97, 1.95, 3.90, 7.81, 15.62, and 31.25 µg/mL. The effect of these synthetic compounds on the growth of the $H_{37}Ra$ strain of *M. tuberculosis* was recorded after 7 days of incubation at 37°C. The observed anti-TB activity revealed that the compounds **3m** (*o*-CH_3), **3e** (*m*-Br), **3b** (*m*-F), and **3c** (*p*-F) were active against the $H_{37}Ra$ strain at all concentrations except 0.97 µg/mL. According to MABA, compounds **3n** (*m*-CH_3), **3q** (*m*-OCH_3), **3i** (*p*-Cl), and **3f** (*p*-Br) were active against the $H_{37}Ra$ strain at concentrations from 7.81-31.25 µg/mL, compounds **3g** (*o*-Cl), **3h** (*m*-Cl), **3d** (*o*-Br), **3o** (*p*-CH_3), and **3p** (*o*-OCH_3) were active at a concentration of 15.62 µg/mL, compounds, **3j** (*o*-NO_2), **3a** (*o*-F), and **3r** (*p*-OCH_3)

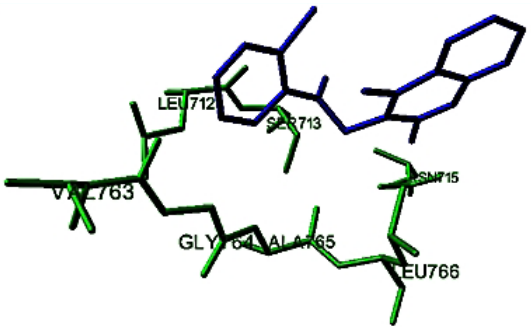

Figure 2. Interaction of the ligand 3-(((2-methylphenyl) amino)methyl)-2-methylquinazolin-4(3*H*)-one 3m receptor and DNA gyrase.

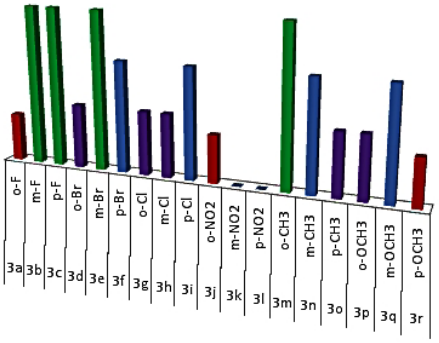

Figure 3. Graph of the MIC of 3-(((substituted phenyl) amino)methyl)-2-methylquinazolin-4(3*H*)-one 3a-3r.

were active at a concentration of 31.25 µg/mL, and compounds **3l** (*p*-NO_2) and **3k** (*m*-NO_2) were inactive at all concentrations (Figure 3). An interesting finding is that compounds with *ortho*, *meta*, or *para* substitutions of electron-accepting groups (fluoro and bromo) and electron-donating groups (methyl and methoxy) had significant activity against the $H_{37}Ra$ strain at all concentrations. The enhanced activity of these compounds means that they are highly potent against microbial strains and should display similar potency against *M. tuberculosis*.

There is an urgent need for the discovery and development of new anti-TB agents that target novel biochemical pathways and treat drug resistant forms of the disease. *M. tuberculosis* is unique in that it is surrounded by a thick and waxy cell wall, so efficient anti-TB drugs should have reasonable lipophilicity to penetrate the cell wall. The screened library contained a set of 3-(((substituted phenyl)amino)methyl)-2-methylquinazolin-4(3*H*)-one compounds, **3a-3r**, that are structurally related to the established drugs bedaquiline, moxifloxacin, gatifloxacin, and ciprofloxacin (Figure 4). The screened compounds consisted of 18 quinazoline analogs with various substituted groups, and most of these compounds were deemed against TB. According to an antimycobacterial bioassay, six of these compounds had significant anti-TB activity, six had modest anti-TB activity, five had slight anti-TB activity, and one was inactive. Table 2 shows the eighteen compounds, **3a-**

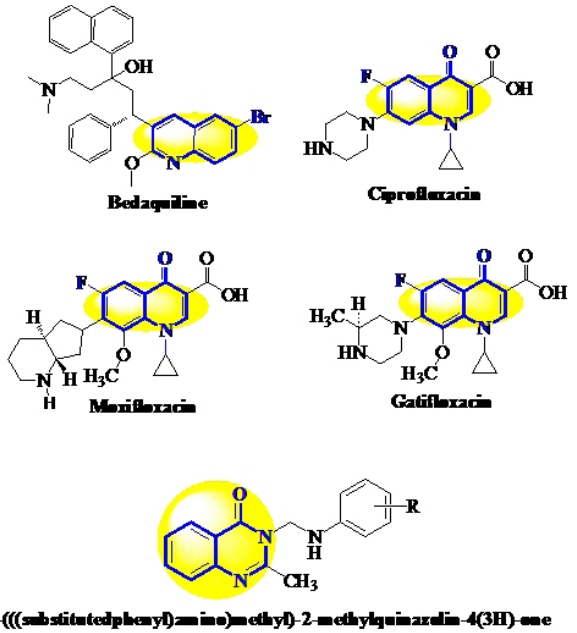

Figure 4. The structures of established drugs and the synthesized 3-(((substituted phenyl) amino) methyl)-2-methylquinazolin-4(3*H*)-one model compound with its pharmacophore features.

3r, with their level of activity against *M. tuberculosis*. Screening revealed that derivatives with *ortho*, *meta*, or *para* substitutions were very similar to the standard anti-TB drug rifampicin. In particular, compounds **3m** (*o*-CH_3), **3e** (*m*-Br), **3b** (*m*-F), and **3c** (*p*-F) had significant anti-TB activity and thus can be considered as potential sources of anti-TB agents. These compounds have all of the hallmarks of a new generation of anti-TB agents along with **3n** (*m*-CH_3), **3q** (*m*-OCH_3), **3i** (*p*-Cl), and **3f** (*p*-Br). Figures 1 and 3 also show a number of interesting aspects of quinazoline that differ from the typical structure associated with different substitutions and anti-TB activity. Compounds with an *ortho*, *meta*, or *para* substitution had significant anti-TB activity, although there were inactive compounds with a *para* or *meta* NO_2 group. All of the tested compounds warrant a thorough discussion of their structure-activity relationship (SAR). There are interesting trends in terms of the activity that was noted. As an example, a quinazoline with a substituted *meta* amine appears to have more potent activity than its close cousin with a substituted *ortho* or *para* amine.

4. Conclusion

In summary, a series of 3-(((substituted phenyl)amino) methyl)-2-methylquinazolin-4(3*H*)-one compounds, **3a-3r**, was synthesized and characterized with IR, ^{1}H NMR, mass spectroscopy, and elemental analyses. Activity was presumably the result of changing the substituents added to the quinazoline core. The presence of *meta*-substituted fluoro, bromo, methyl, methoxy, or chloro groups on the aromatic amine ring increased the activity of compounds in comparison to compounds with other substituents. A search of the literature for this specific scaffold yielded no previous reports in relation to TB. The current results shed light on how quinazoline analogs with different substituted groups display different levels of activity. In light of the above findings regarding the effect of certain scaffolds on activity, the mode of action of the studied compounds should be determined and analogs should be prepared to develop a clear picture of their SAR. The analogs synthesized here will benefit future studies of quinazoline by chemists and researchers.

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Appendix
Spectral data for the synthesized compounds

2-Methyl-4H-benzo[1,3]oxazin-4-one (1) IR (KBr) cm^{-1}: 3,000 (Ar-CH str), 2,942 (CH_3-CH str), 1,715 (C=O), 1,636 (C=N), 1,057 (C-O-Cstr);^{1}H-NMR (300 MHz, $CDCl_3$): δppm: 2.32 (s, 3H, CH_3), 6.98-7.47 (m, 4H, Ar-H); MS (EI) *m/z*: 161 [M+]; Anal. Calcd for $C_9H_7NO_2$: C, 67.07; H, 4.38; N, 8.69. Found: C, 67.16; H, 4.40; N, 8.66.

2-methylquinazolin-4(3H)-one (2) IR (KBr) cm^{-1}: 3,229 (NH str), 3,012 (Ar-CH str), 2,882 (CH_3-CH str), 1,710 (C=O), 1,629 (C=N); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.41 (s, 3H, CH_3), 6.92-7.40 (m, 4H, Ar-H), 9.31 (s, 1H, NH); MS (EI) *m/z*: 160 [M+]; Anal. Calcd for $C_9H_8N_2O$: C, 67.49; H, 5.03; N, 17.49. Found: C, 67.41; H, 5.09; N, 17.42.

3-(((2-fluorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3a) IR (KBr) cm^{-1}: 3,385 (NH str), 3,035 (Ar-CH str), 2,970 (CH_3-CH str), 1,700 (C=O), 1,009 (C-F);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.12 (s, 3H, CH_3), 4.55 (s, 2H, CH2), 7.12-7.50 (m, 8H, Ar-H), 8.48 (s, 1H, NH); MS (EI) *m/z*: 283 [M+]; Anal. Calcd for $C_{16}H_{14}FN_3O$: C, 67.83; H, 4.98; N, 14.83. Found: C, 67.82; H, 4.91; N, 14.86.

3-(((3-fluorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3b) IR (KBr) cm^{-1}: 3,397 (NH str), 3,013 (Ar-CH str), 2,921 (CH_3-CH str), 1,711 (C=O), 1101 (C-F);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.92 (s, 3H, CH_3), 4.27 (s, 2H, CH2), 7.52-8.18 (m, 8H, Ar-H), 9.24 (s, 1H, NH); MS (EI) *m/z*: 283 [M+]; Anal. Calcd for $C_{16}H_{14}FN_3O$: C, 67.83; H, 4.98; N, 14.83. Found: C, 67.86; H, 4.97; N, 14.87.

3-(((4-fluorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3c) IR (KBr) cm^{-1}: 3,387 (NH str), 3,082 (Ar-CH str), 2,927 (CH_3-CH str), 1,708 (C=O), 1,031 (C-F);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.44 (s, 3H, CH_3), 4.47 (s, 2H, CH2), 7.12-8.10 (m, 8H, Ar-H), 8.94 (s, 1H, NH); MS (EI) *m/z*: 283 [M+]; Anal. Calcd for $C_{16}H_{14}FN_3O$: C, 67.83; H, 4.98; N, 14.83. Found: C, 67.87; H, 4.95; N, 14.81.

3-(((2-bromophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3d) IR (KBr) cm^{-1}: 3,344 (NH str), 3,020 (Ar-CH str), 2,842 (CH_3-CH str), 1,702 (C=O), 642 (C-Br);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.17 (s, 3H, CH_3), 4.25 (s, 2H, CH2),6.92-7.86 (m, 8H, Ar-H), 9.14 (s, 1H, NH); MS (EI) *m/z*: 346 [M+2]; Anal. Calcd for $C_{16}H_{14}BrN_3O$: C, 55.83; H, 4.10; N, 12.21. Found: C, 55.85; H, 4.13; N, 12.24.

3-(((3-bromophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3e) IR (KBr) cm^{-1}: 3,417 (NH str), 3,003 (Ar-CH str), 2,901 (CH_3-CH str), 1,706 (C=O), 598 (C-Br); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.42 (s, 3H, CH_3), 4.12 (s, 2H, CH2), 7.12-7.28 (m, 8H, Ar-H), 8.94 (s, 1H, NH); MS (EI) *m/z*: 346 [M+2]; Anal. Calcd for $C_{16}H_{14}BrN_3O$: C, 55.83; H, 4.10; N, 12.21. Found: C, 55.84; H, 4.11; N, 12.21.

3-(((4-bromophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3f) IR (KBr) cm^{-1}: 3,407 (NH str), 3,009 (Ar-CH str), 2,900 (CH_3-CH str), 1,712 (C=O), 627 (C-Br);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.02 (s, 3H, CH_3), 4.12 (s, 2H, CH2), 7.12-7.90 (m, 8H, Ar-H), 8.99 (s, 1H, NH); MS (EI) *m/z*: 346 [M+2]; Anal. Calcd for $C_{16}H_{14}BrN_3O$: C, 55.83; H, 4.10; N, 12.21. Found: C, 55.78; H, 4.15; N, 12.28.

3-(((2-chlorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3g) IR (KBr) cm^{-1}: 3,403 (NH str), 3,005 (Ar-CH str), 2,909 (CH_3-CH str), 1,712 (C=O), 722 (C-Cl);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.22 (s, 3H, CH_3), 4.35 (s, 2H, CH2), 7.22-7.70 (m, 8H,

Ar-H), 9.48 (s, 1H, NH); MS (EI) *m/z*: 301 [M+2]; Anal. Calcd for $C_{16}H_{14}ClN_3O$: C, 64.11; H, 4.71; N, 14.02. Found: C, 64.15; H, 4.76; N, 14.00.

3-(((3-chlorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3h) IR (KBr) cm^{-1}: 3,377 (NH str), 3,011 (Ar-CH str), 2,901 (CH_3-CH str), 1,701 (C=O), 714 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.32 (s, 3H, CH_3), 4.47 (s, 2H, CH2), 7.32-8.02 (m, 8H, Ar-H), 9.14 (s, 1H, NH); MS (EI) *m/z*: 301 [M+2]; Anal. Calcd for $C_{16}H_{14}ClN_3O$: C, 64.11; H, 4.71; N, 14.02. Found: C, 64.13; H, 4.73; N, 14.06.

3-(((4-chlorophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3i) IR (KBr) cm^{-1}: 3,417 (NH str), 3,012 (Ar-CH str), 2,907 (CH_3-CH str), 1,705 (C=O), 742 (C-Cl); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.47 (s, 3H, CH_3), 4.42 (s, 2H, CH2), 7.22-8.40 (m, 8H, Ar-H), 9.11 (s, 1H, NH); MS (EI) *m/z*: 301 [M+2]; Anal. Calcd for $C_{16}H_{14}ClN_3O$: C, 64.11; H, 4.71; N, 14.02. Found: C, 64.09; H, 4.70; N, 14.03.

3-(((2-nitrophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3j) IR (KBr) cm^{-1}: 3,412 (NH str), 3,000 (Ar-CH str), 2,889 (CH_3-CH str), 1,707 (C=O), 1,502 (N-O);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 3.11 (s, 3H, CH_3), 4.51 (s, 2H, CH2), 7.11-7.51 (m, 8H, Ar-H), 8.41 (s, 1H, NH); MS (EI) *m/z*: 310 [M+]; Anal. Calcd for $C_{16}H_{14}N_4O_3$: C, 61.93; H, 4.55; N, 18.06. Found: C, 61.97; H, 4.58; N, 18.02.

3-(((3-nitrophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3k) IR (KBr) cm^{-1}: 3,497 (NH str), 3,014 (Ar-CH str), 2,941 (CH_3-CH str), 1,714 (C=O), 1,542 (N-O);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.94 (s, 3H, CH_3), 4.24 (s, 2H, CH2), 7.54-8.14 (m, 8H, Ar-H), 9.44 (s, 1H, NH); MS (EI) *m/z*: 310 [M+]; Anal. Calcd for $C_{16}H_{14}N_4O_3$: C, 61.93; H, 4.55; N, 18.06. Found: C, 62.00; H, 4.60; N, 18.10.

3-(((4-nitrophenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3l) IR (KBr) cm^{-1}: 3,337 (NH str), 3,032 (Ar-CH str), 2,937 (CH_3-CH str), 1,703 (C=O), 1,498 (N-O);^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.34 (s, 3H, CH_3), 4.43 (s, 2H, CH2), 7.13-8.30 (m, 8H, Ar-H), 8.93 (s, 1H, NH); MS (EI) *m/z*: 310 [M+]; Anal. Calcd for $C_{16}H_{14}N_4O_3$: C, 61.93; H, 4.55; N, 18.06. Found: C, 61.92; H, 4.51; N, 18.07.

3-(((2-methylphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3m) IR (KBr) cm^{-1}: 3,415 (NH str), 3,015 (Ar-CH str), 2,910 (CH_3-CH str), 1,710 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.42 (s, 3H, CH_3), 3.12 (s, 3H, CH_3), 4.51 (s, 2H, CH2), 7.11-7.51 (m, 8H, Ar-H), 8.41 (s, 1H, NH); MS (EI) *m/z*: 279 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O$: C, 73.10; H, 6.13; N, 15.04. Found: C, 73.11; H, 6.14; N, 15.01.

3-(((3-methylphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3n) IR (KBr) cm^{-1}: 3,337 (NH str), 3,033 (Ar-CH str), 2,931 (CH_3-CH str), 1,713 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.32 (s, 3H, CH_3), 2.92 (s, 3H, CH_3), 4.23 (s, 2H, CH2), 7.32-8.13 (m, 8H, Ar-H), 9.34 (s, 1H, NH); MS (EI) *m/z*: 279 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O$: C, 73.10; H, 6.13; N, 15.04. Found: C, 73.15; H, 6.15; N, 15.07.

3-(((4-methylphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3o) IR (KBr) cm^{-1}: 3,385 (NH str), 3,052 (Ar-CH str), 2,925 (CH_3-CH str), 1,705 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.45 (s, 3H, CH_3), 3.45 (s, 3H, CH_3), 4.57 (s, 2H, CH2), 7.52-8.15 (m, 8H, Ar-H), 8.95 (s, 1H, NH); MS (EI) *m/z*: 279 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O$: C, 73.10; H, 6.13; N, 15.04. Found: C, 73.13; H, 6.17; N, 15.09.

3-(((2-methoxyphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3p) IR (KBr) cm^{-1}: 3,375 (NH str), 3,003 (Ar-CH str), 2,911 (CH_3-CH str), 1,714 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.45 (s, 3H, CH_3), 3.17 (s, 3H, OCH_3), 4.56 (s, 2H, CH2), 7.10-7.74 (m, 8H, Ar-H), 8.49 (s, 1H, NH); MS (EI) *m/z*: 295 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O_2$: C, 69.14; H, 5.80; N, 14.23. Found: C, 69.19; H, 5.81; N, 14.27.

3-(((3-methoxyphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3q) IR (KBr) cm^{-1}: 3,431 (NH str), 3,009 (Ar-CH str), 2,907 (CH_3-CH str), 1,701 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.37 (s, 3H, CH_3), 3.12 (s, 3H, OCH_3), 4.47 (s, 2H, CH2), 7.12-7.73 (m, 8H, Ar-H), 9.12 (s, 1H, NH); MS (EI) *m/z*: 295 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O_2$: C, 69.14; H, 5.80; N, 14.23. Found: C, 69.11; H, 5.83; N, 14.21.

3-(((4-methoxyphenyl)amino)methyl)-2-methylquinazolin-4(3H)-one (3r) IR (KBr) cm^{-1}: 3,415 (NH str), 3,031 (Ar-CH str), 2,911 (CH_3-CH str), 1,701 (C=O); ^{1}H-NMR (300 MHz, $CDCl_3$): δ ppm: 2.31 (s, 3H, CH_3), 2.95 (s, 3H, OCH_3), 4.51 (s, 2H, CH2), 7.44-7.97 (m, 8H, Ar-H), 8.71 (s, 1H, NH); MS (EI) *m/z*: 295 [M+]; Anal. Calcd for $C_{17}H_{17}N_3O_2$: C, 69.14; H, 5.80; N, 14.23. Found: C, 69.19; H, 5.81; N, 14.25.

Original Article

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Dividing phase-dependent cytotoxicity profiling of human embryonic lung fibroblast identifies candidate anticancer reagents

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Summary **Human Embryonic Lung fibroblasts (HEL cells) are widely used as a normal cell in studies of cell biology and can be easily maintained in the resting phase. Here we aimed to discover compounds that exhibit cytotoxicity against HEL cells in the dividing phase, but not in the resting phase. The cytotoxicity of each compound against HEL cells either in the resting phase or in the dividing phase was determined by MTT assay. Ratios of the IC50 of cells in the resting phase and that of cells in the dividing phase (RRD) for these compounds were compared. We selected 44 compounds that exhibited toxic effects on HEL cells in the dividing phase from a chemical library containing 325 anticancer drugs and enzyme inhibitors. The RRD values of those compounds were widely distributed. Paclitaxel and docetaxel, which are clinically used as anticancer drugs, had RRD values larger than 2000. On the other hand, the RRD value of dimethyl sulfoxide, an organic solvent, was 1. The cytotoxic effect of paclitaxel on HEL cells in the dividing phase was attenuated by aphidicolin, hydroxyurea, and nocodazole, confirming that the cytotoxic effects of paclitaxel are dependent on cells being in the dividing phase. Thapsigargin, whose RRD value was 800, the third highest RRD value in the library, exhibited therapeutic effects in a mouse model of FM3A ascites carcinoma. We suggest that compounds with high RRD values for HEL cells are candidate anticancer chemotherapy seeds.**

Keywords: Dividing-phase cell, resting-phase cell, anti-cancer reagent, screening, human embryonic lung fibroblast, thapsigargin

1. Introduction

For the development of novel anticancer drugs, chemicals with low cytotoxic effects in normal cells compared with cancer cells are generally screened in the first stage. Almost all chemicals screened based on this characteristic, however, are dropped during drug development at the preclinical stage, resulting in a limited number of candidates available for evaluation at the clinical stages. Overcoming this problem requires the establishment of novel strategies to screen candidate compounds for cancer chemotherapies.

Most of the cells in the bodies of the developed organism are in resting phase (*1,2*). In contrast, cancer cells are always in dividing phase due to mutations in genes related to the regulation of cell division (*3*). The principles of selective toxicity of anticancer reagents are generally based on the assumption that cancer cells are in the dividing stage whereas normal cells are typically in the resting stage. Clinically applied anticancer drugs have severe toxicity against normal cells in the dividing stage (*4*), and therefore strict caution is required when administering anticancer drugs to patients. Even chemicals with some cytotoxic effects on normal cells, however, may be clinically useful as anticancer drugs. In the present study, we hypothesized that compounds with potent cytotoxicity against normal cells in the dividing phase but no cytotoxic effects against normal cells in the resting phase might be effective anticancer drugs. In other words, we considered that comparing cytotoxicity of compounds against dividing-phase and resting-phase

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normal cells would be effective for screening candidate anticancer drugs.

Human embryonic lung fibroblasts (HEL cells) are widely used as normal cells in studies of general cell biology. This cell line forms a cell sheet where contact inhibition is induced when the cells are cultured under conditions of low serum concentration (*5*). Therefore, HEL cells in the resting phase can be easily prepared on a relatively large scale (*6,7*). In the present study, we screened compounds with cytotoxicity against dividing-phase HEL cells but not against resting-phase HEL cells. Our findings indicated that paclitaxel and docetaxel, which are clinically applied as anticancer drugs have selective cytotoxicity to HEL cells in the dividing stage. Thapsigargin also exhibited selective cytotoxicity to HEL cells in the dividing stage and had therapeutic effects in a mouse model of FM3A ascites carcinoma.

2. Materials and Methods

2.1. *Cells and reagents*

HEL cells were provided by Dr. Yasushi Kawaguchi in the Division of Molecular Virology, Department of Microbiology and Immunology, at the Institute of Medical Science, the University of Tokyo, Tokyo, Japan. The mouse mammary cancer cell line FM3A was provided by Dr. Fumio Hanaoka at the Institute for Biomolecular Science, Faculty of Science, Gakushuin University, Tokyo, Japan. An inhibitor kit containing 323 chemicals was provided by the Screening Committee of Anticancer Drugs (director; Dr. Takao Yamori). Paclitaxel, docetaxel, thapsigargin, cisplatin, cycloheximide, cytochalasin D, lovastatin, camptothecin, aphidicolin, and nocodazole were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxyurea was purchased from Nacalai Tesque (Kyoto Japan). SB225002 and radicicol were purchased from Calbiochem, San Diego, CA, USA. Bortezomib was purchased from Selleck Chemicals, Houston, TX, USA. As reagents for cell culture, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 0.25% Trypsin/EDTA solution were purchased from Nacalai Tesque, Hyclone (Logan, UT), and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. *Cytotoxicity assay using HEL cells in vitro*

The cytotoxicity of each compound against HEL cells was evaluated by MTT assay (Figure 1A). HEL cells were cultured in DMEM supplemented with 10% FBS. Cells (4×10^3) were seeded on 96-well plates and incubated at 37˚C for 12 h, prepared as dividing-phase HEL cells. Cells were further incubated with the same medium containing each compound for 2 days. Thiazolyl blue tetrazolium bromide was added and incubation was continued for 4 h. Then, cells were solubilized, and absorbance at 595 nm was measured.

For preparation of HEL cells in the resting stage, cells (4×10^4 cells) were seeded on 96-well plates and monolayer cell sheets were produced by incubation for 12 h. Cells were further cultured in DMEM containing 1% FBS for 7 days and prepared as resting-phase HEL cells. Then, cells were cultured with the same medium containing each compound for 2 days and subjected to the MTT assay as described above.

To evaluate cytotoxicity during the dividing phase or resting phase, HEL cells were cultured with chemicals from a library at concentrations of 0.02 μM and 2 μM.

2.3. *Anti-tumor assay using a mouse model of FM3A ascites carcinoma*

FM3A cells were suspended in PBS, and 1×10^6 cells were transplanted into the peritoneal cavity of 5-week-old female C3H/He-JJ mice. Starting the next day, thapsigargin (56 mg/kg) or PBS was injected intraperitoneally every day and the survival period was determined by daily observation.

2.4. *Statistical analysis*

All experiments in the present study were performed at least twice. The data are shown as mean ± standard error of the mean (SEM) and significant differences were evaluated by Student's *t*-test.

3. Results

3.1. *Chemicals with cytotoxic effects against HEL cells in the dividing-phase*

We aimed to establish a system for screening compounds with cytotoxicity against HEL cells in the dividing-phase, but not in the resting-phase. HEL cells in the dividing-phase and resting-phase were prepared by changing the concentration of serum in the culture medium. A schematic protocol for screening compounds that are cytotoxic against dividing-phase cells, but not against resting-phase cells is shown in Figure. 1A. First, we selected compounds from our chemical library containing 325 cell growth-inhibiting compounds that exhibited cytotoxicity against HEL cells in the dividing-phase, including current clinically applied anticancer drugs. We identified 44 chemicals that exhibited cytotoxic effects at a concentration of 2 μM against HEL cells in the dividing phase (Table S1, *http://ddtjournal.com/docindex.php?year=2016&kanno=4*). Paclitaxel (PTX) exhibited cytotoxic effects against dividing-phase HEL cells at 0.001 μM, but not against HEL cells in the resting-phase, even at 10 μM (Figure. 1B, 1C). In contrast, dimethyl sulfoxide (DMSO), an organic solvent, exhibited cytotoxic effects against both dividing-phase and resting-phase HEL cells at the

same concentration of 700 mM. We used the ratio of the IC_{50} for dividing-phase HEL cells to the IC50 for resting-phase HEL cells as a criterion of specificity of the cytotoxic effects against dividing-phase cells. This value was defined as the ratio between the resting phase

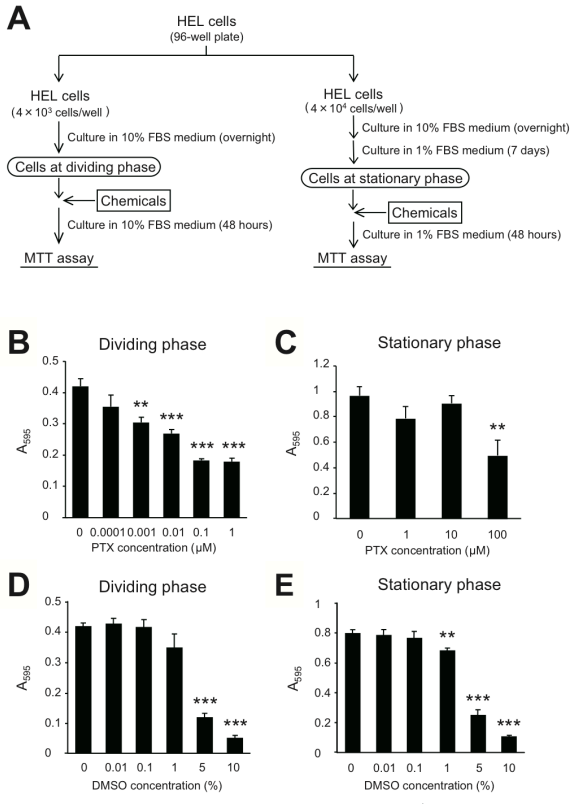

Figure 1. Identification of cytotoxic compounds that act specifically on HEL cells in the dividing-phase. (A) Protocol for preparation of dividing-phase and resting-phase HEL cells and for the chemical cytotoxicity tests. Cytotoxicity of paclitaxel against dividing-phase **(B)** and resting-phase HEL cells **(C)**. Cytotoxicity of DMSO against dividing-phase **(D)** and resting-phase HEL cells **(E)**. HEL cells were cultured with the chemicals for 48 h, followed by MTT assay. Statistically significant differences were evaluated by comparing A_{595} values of the chemicals with those of the controls (without chemicals). $^{**}p < 0.01$, $^{***}p < 0.001$

IC_{50} and the dividing phase IC_{50} (RRD). RRD values of all compounds tested were larger than 1. We considered that compounds with a higher RRD value would have more potent cytotoxicity against dividing-phase HEL cells than resting-phase HEL cells. Eight chemicals in the present chemical library had RRD values larger than 10 (Figure. 2 and Table. 1). In particular, RRD values of docetaxel, paclitaxel, and thapsigargin were 6,000, > 2,000, and 750, respectively. In contrast, DMSO had the same RRD value (RRD = 1) against dividing-phase and resting-phase HEL cells. This means that RRD values widely differ between chemicals.

3.2. *Effect of cell division inhibitors on the cytotoxicity of paclitaxel against HEL cells in the dividing-phase*

We considered that compounds with high RRD values would have selective toxicity against dividing-phase HEL

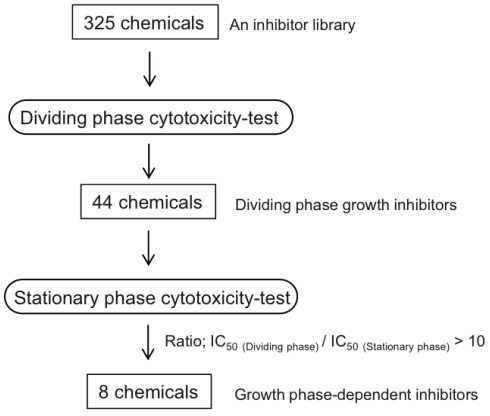

Figure 2. Selection of compounds exhibiting selective toxicity against HEL cells in the dividing phase. First, 44 compounds were selected as growth inhibitors of dividing-phase HEL cells. Next, the IC_{50} values of the compounds against resting-phase HEL cells were determined. RRD values (proportion of IC_{50} for resting-phase HEL cells to that for dividing-phase HEL cells) were calculated. RRD values of 8 compounds were greater than 10.

Table 1. Cytotoxic effect of inhibitors to HEL cell

Chemicals	Target	Dividing phase IC_{50}* (μM)	Resting phase IC_{50}* (μM)	Ratio (resting/dividing)
Paclitaxel	Tubulin depolymerization	0.05	> 100	> 2,000
Docetaxel	Tubulin depolymerization	0.01	60	6,000
Thapsigargin	Ca^{2+} ATPase	0.008	6	750
Cycloheximide	Protein synthesis	1.6	340	213
Cytochalasin D	Actin filamentation	0.06	5.5	92
SB225002	CXCR2	1.8	36	20
Lovastatin	HMG-CoA reductase	7.5	100	13
PDGF receptor tyrosine kinase inhibitor IV	PDGF receptor	0.2	> 2	> 10
Bortezomib	Proteasome	0.2	1.3	7
Cisplatin	DNA replication	23	120	5
Radicicol	Hsp90	3	13	4
Camptothecin	DNA topoisomerase	1.1	2.0	2
DMSO		400,000	400,000	1

*IC_{50} was defined as the concentration of each chemical, which shows 50% inhibition of cell viability determined by MTT assay. Control cells were treated with DMSO, a solvent for chemicals.

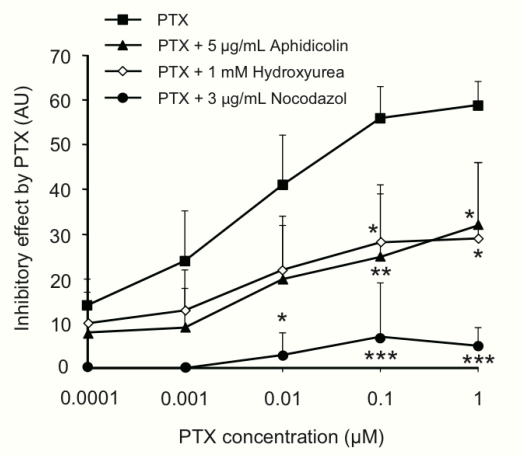

Figure 3. Attenuation of the cytotoxicity of paclitaxel by pre-treatment of HEL cells with cell-cycle inhibitors. HEL cells in the dividing-phase were prepared. The cells were then incubated with paclitaxel and cell-cycle inhibitors. Cell viability was determined by MTT assay. The cell growth inhibitory effects of paclitaxel (AU) were calculated by (A_{595} [without paclitaxel] – A_{595} [with paclitaxel])/A_{595} [without paclitaxel]. Significant differences were evaluated compared with control (without cell-cycle inhibitors). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2. Change of IC50 values of chemicals by inhibitors of cell cycle

Chemicals	IC_{50}
Paclitaxel	0.05 μM
Paclitaxel + Nocodazole 3 μg/mL	> 1 μM
Paclitaxel + Colchicine 5 μg/mL	> 1 μM
Paclitaxel + Aphidicolin 5 μg/mL	> 1 μM
Paclitaxel + Hydroxyurea 1 mM	> 1 μM
DMSO	400 mM
DMSO + Nocodazole 3 μg/mL	600 mM
DMSO + Aphidicolin 5 μg/mL	800 mM
DMSO + Hydroxyurea 1 mM	400 mM

Dividing phase HEL cells were treated with paclitaxel or DMSO in the absence or presence of cell cycle inhibitors. IC_{50} values of paclitaxel or DMSO were determined.

cells, but not against resting-phase HEL cells. If this is correct, these compounds would only have toxic effects if the cells were in the dividing phase. In other words, the cytotoxic effects of such compounds would be expected to be reduced if cell division was inhibited. Therefore, we tested whether the cytotoxicity of paclitaxel, which has an RRD value larger than 2,000, would be attenuated by the addition of cell-division inhibitors. Aphidicolin, hydroxyurea, and nocodazole suppress cell division *via* the inhibition of DNA polymerase α, nucleoside dehydrogenase, and tubulin polymerization, respectively (*8-10*). Each inhibitor was added to halt cell division of the dividing-phase HEL cells and the cytotoxic effect of paclitaxel was examined by MTT assay. The cytotoxic effect of paclitaxel was suppressed by these cell division inhibitors (Figure. 3 and Table. 2), indicating that paclitaxel exhibits cytotoxicity only against cells in the

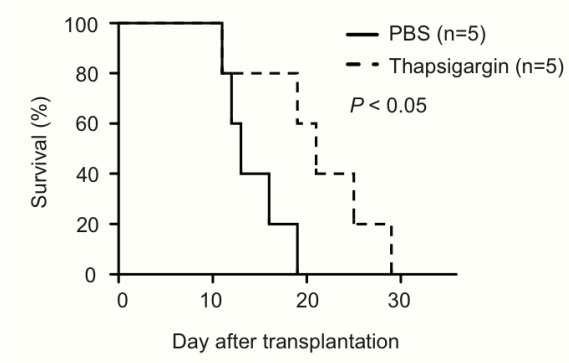

Figure 4. Anticancer effect of thapsigargin in a mouse model of FM3A ascites carcinoma. FM3A cells were continuously cultured *in vitro* and suspended in PBS (-). The cells (1×10^6) were transplanted into the peritoneal cavity of 5-week-old female C3H/He-JJ mice. Starting the next day, 56 mg/kg thapsigargin or PBS (-) was injected intraperitoneally every day and the number of surviving mice was counted.

dividing phase. On the other hand, the cytotoxic effect of DMSO was not suppressed by treatment with cell division inhibitors (Figure. S1, *http://ddtjournal.com/docindex.php?year=2016&kanno=4*), thus cytotoxicity of DMSO does not require cells to be in the dividing phase.

3.3. *Anticancer effect of thapsigargin*

We assumed that chemicals with a higher RRD value would have less cytotoxicity against normal cells although they exhibited potent toxicity against cancer cells. Paclitaxel (RRD = 6,000) and docetaxel (RRD > 2,000) are already used as anticancer drugs for human patients (*11*). Because the RRD value of thapsigargin (RRD = 750) was much higher than that of other chemicals in the library, we examined whether thapsigargin would exhibit anticancer effects in a mouse tumor model. Our findings demonstrated that repeated administration of thapsigargin conferred a survival advantage to a mouse model of FM3A ascites carcinoma compared to mice administered PBS (Figure. 4).

4. Discussion

In the present study, we established a screening system for detecting compounds with potent cytotoxic effects against HEL cells in the dividing phase but not against those in the resting phase. We identified several compounds with cytotoxic effects in dividing-phase HEL cells at low concentrations, but not in resting phase HEL cells. Furthermore, we demonstrated that thapsigargin, which exhibited selective cytotoxic effects on dividing-phase HEL cells, prolonged survival in a mouse model of ascites carcinoma.

Recently, the development of anticancer drugs with molecular targets has gained popularity (*12-14*). Only a few compounds selected by this method have

inhibitory effects on cancer cell growth without toxicity to normal cells (*15*). In contrast, the classical concept of cell growth inhibitors as anticancer drugs is associated with strong side effects, although they are highly effective for growth inhibition of cancer cells. Thus, the number of compounds that can be clinically applied as anticancer drugs is currently limited (*16*). In the present study, we focused on the finding that a large proportion of intravital normal cells are in the resting phase. We defined the ratio of the compound concentration with cytotoxic effects on HEL cells in the dividing phase and those in the resting phase as the RRD value, and established a method for screening compounds with high RRD values. Screening of our chemical library revealed that docetaxel and paclitaxel, which are clinically used as anticancer drugs, had strong cytotoxic effects on dividing-phase cells, but not on resting-phase cells, resulting in high RRD values (> 2,000).

We also demonstrated that several compounds not previously considered to be candidate anticancer drugs had more potent cytotoxic effects on dividing-phase cells than on resting-phase cells. In particular, thapsigargin was identified as a compound with a high RRD value. Thapsigargin is isolated from *Thapsia garganica* and used as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) (*17-19*). Thapsigargin is suggested to have cytotoxic effects on cancer cells *in vitro via* the induction of endoplasmic reticulum stress (*20,21*). Endoplasmic reticulum stress inducers like thapsigargin have cytotoxic effects on cancer cells with multidrug resistance (*22,23*). In the present study, we demonstrated that thapsigargin confers a survival advantage to a mouse model of FM3A ascites carcinoma. This finding suggests that compounds with a high RRD value have potential as candidate anticancer drugs although *in vivo* trials are necessary for analyzing the anti-cancer effect and toxicity to normal proliferative cells such as bone marrow cells. The evaluation system proposed here will facilitate the discovery of seeds for clinically applicable anticancer drugs.

Chemical screening using our established system revealed eight chemicals in the chemical library with significant cytotoxic effects on HEL cells in the dividing phase. Thapsigargin (RRD = 750) treatment prolonged survival in a mouse model of FM3A ascites carcinoma. These findings suggest that this method of screening compounds with high RRD values using HEL cells is applicable for identifying candidate anticancer drugs. The new screening strategy of anticancer drug established in the present study will increase the number of candidate anticancer drugs.

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Original Article

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In vitro evaluation of the antiviral activity of methylglyoxal against influenza B virus infection

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Summary **Influenza A and B virus infections are serious public health concerns globally. However, the concerns regarding influenza B infection have been underestimated. The currently used anti-influenza drugs have not provided equal efficacy for both influenza A and B viruses. Susceptibility to neuraminidase (NA) inhibitors has been observed to be lower for influenza B viruses than for influenza A viruses. Moreover, the emergence of resistance to anti-influenza drugs underscores the need to develop new drugs. Recently, we reported that methylglyoxal (MGO) suppressed influenza A virus replication in a strain-independent manner. Therefore, we hypothesize that MGO exhibits anti-influenza activity against B strains. This study aimed to evaluate the anti-influenza viral activity of MGO against influenza B strains by using Madin-Darby canine kidney (MDCK) cells. Several types of influenza B viruses were used to determine the activity of MGO. The susceptibilities of influenza A and B viruses to NA inhibitors were compared. MGO inhibited influenza B virus replication, with 50% inhibitory concentrations ranging from 23-140 μM, which indicated greater sensitivity of influenza B viruses than influenza A viruses. Our results show that MGO has potent inhibitory activity against influenza B viruses, including NA inhibitor-resistant strains.**

Keywords: Influenza virus, anti-influenza viral drug, methylglyoxal, neuraminidase inhibitors

1. Introduction

Influenza viruses are enveloped, negative, single-stranded RNA viruses with eight segmented genomes belonging to the *Orthomyxoviridae* family. There are three distinct virus types, A, B, and C, distinguished according to their antigenicity to internal protein structures, nucleoprotein (NP) and matrix protein. Influenza A and B viruses are important human respiratory pathogens that cause epidemics with significant disease burden. Although concern regarding influenza B virus infection relative to influenza A in humans has been neglected in the past, recent studies have shown that influenza B infection causes similar rates of mortality in some epidemic seasons, especially in children (*1,2*). Clinical reports also have shown that infection by influenza B viruses tends to induce lethal secondary bacterial infections and myocardial or neurological complications (*3-6*). Although influenza B viruses share similar fundamental structural features of this family, these have different characteristics from those of influenza A viruses; for example, the matrix BM2 has been found to have ion channel activity equal to that of influenza A viruses M2 (*7*) . Moreover, the influenza A virus shows a more rapid rate of evolution than that of the influenza B virus, which lacks a wild animal reservoir (*8*).

During the last step of the virus life cycle, neuraminidase (NA) plays an important role in removal of sialic acid from cellular receptors recognized by hemagglutinin (HA), which results in the release of new progeny virions from infected cells. Because the HAs of neighboring virions recognize and bind to neuraminic acid residues, which cause self-aggregation of new progeny virions, release of new virions, therefore, requires the receptor-destroying activity of NA to cleave

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glycoconjugates between viral glycoproteins and host cell molecules (*9,10*). NA was chosen as a suitable drug target because it has a critical role in the influenza life cycle. Amino acid residues of the catalytic site or the framework of the enzyme are highly conserved in both influenza type A and B (*11,12*).

NA inhibitors (NAIs) were designed to be sialic acid analogues that potently and specifically inhibit influenza virus replication by competitively binding to the NA active site, which results in inhibition of cleavage of the cell surface and prevention of the release of newly formed virions (*13*). There are currently three Food and Drug Administration-approved drugs effective for influenza B virus worldwide, oseltamivir (Tamiflu®, Roche), zanamivir (Relenza®, GlaxoSmithKline), and peramivir (Rapivab®, Biocryst). Inhaled laninamivir (Inavir®, Daiichi-sankyo) is also approved in Japan.

Several studies have reported that NAIs may have a lower efficacy against influenza B viruses than against influenza A (*9,14-16*). *In vitro* studies also have shown that the 50% inhibitory concentrations (IC_{50}) of oseltamivir were dramatically higher for influenza B than for influenza A viruses (*17*). The elevated IC_{50} of oseltamivir for influenza B may result from the structure of NA protein that is less flexible than that of influenza A, which causes incomplete binding to the hydrophobic pocket of oseltamivir (*18*). The susceptibilities of NAIs have been considered to be dependent on the B lineage in the same manner as observed for different influenza A neuraminidase subtypes (*14*).

Natural products, such as microbial metabolites and medicinal plants, are promising as potentially effective and novel antiviral drugs. To date, several agents isolated from these natural products have been reported. We found that manuka honey exhibited the highest anti-influenza activity among tested honey samples (*19*). We recently reported that the α-ketoaldehyde compound, methylglyoxal (MGO; molecular weight 72.06; Figure 1), which is present in extremely high concentrations in manuka honey (*20*), has potent inhibitory activity against multiple influenza A subtypes, including the oseltamivir-resistant influenza strain. The mechanism of MGO is thought to involve direct interaction of MGO on the virus surface and interference with the interactions between viruses and host cells. Because the mode of action of MGO is different from that of NAIs, MGO in combination with NAI could enhance the NAI activity for inhibition of influenza A virus replication (*21*).

In this study, we compared the anti-influenza viral activity of MGO against influenza B viruses with that of other NAIs and evaluated its potential as a new universal antiviral agent against influenza viruses. In addition, we also compared the susceptibilities to NAIs of several strains of influenza B virus, including laboratory strains and clinically isolated samples from patients in Japan, with those of influenza A viruses reported previously (*21*). MGO exhibited a broad spectrum of inhibitory activity against influenza B viruses, not only against NAI-sensitive influenza virus B strains, but also against NAI-resistant influenza B strains. Moreover, influenza B viruses were found to be more sensitive to MGO than were influenza A viruses, as shown by lower IC_{50s} against influenza B viruses than against influenza A viruses.

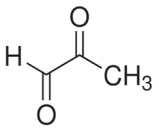

Figure 1. Chemical structure of MGO.

2. Materials and Methods

2.1. *Cells, viruses and chemicals*

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (E-MEM) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) supplemented with 5% fetal bovine serum (FBS) purchased from Sigma-Aldrich (St. Louis, MO, USA) at 37°C in an atmosphere of 5% CO_2. B/Lee/40 (ATCC® VR-1535™) and B/Brisbane/60/2008 were purchased from the American Tissue Culture collection (Manassas, Virginia, USA) and National Institute of Infectious Diseases (Tokyo, Japan), respectively. Influenza B clinical strains were isolated in our laboratory from clinical specimens collected from a hospital in Japan during 2014. Influenza virus B strains used in this study were propagated in MDCK cells. The 50% tissue culture infective dose ($TCID_{50}$) of influenza virus was titrated by using MDCK cells. Oseltamivir, zanamivir, peramivir and laninamivir were purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland), GlaxoSmithKline PLC (Middlesex, UK), Biocryst, Inc. (Durham, NC, USA), and Daiichi Sankyo, Ltd. (Tokyo, Japan), respectively. Oseltamivir was dissolved in H_2O to a concentration of 24 mM, and zanamivir and laninamivir were dissolved in dimethyl sulphoxide (CultureSure® DMSO) purchased from Wako Pure Chemical Industries, Ltd. to concentrations of 25 mM and 564.34 mM, respectively. Peramivir (30.5 mM) was directly used without dilution. All samples were maintained at -80°C. Approximately 40% MGO solution (in H_2O) was purchased from Sigma-Aldrich and maintained at 4°C. Prior to performing experiments, MGO was diluted with E-MEM supplemented with 1% 100× vitamin solution (MEM-vitamin) purchased from Gibco® (Carlsbad, CA, USA).

2.2. *Evaluation of anti-influenza viral and cytotoxic activities*

The anti-influenza viral activities of MGO and NAIs

were evaluated as previously described (*21*), with slight modifications. For evaluation of anti-influenza viral activities, MDCK cells were typically seeded in 96-well plates at a density of 3.0×10^4 cells/well in 100 µL of MEM containing 10% FBS and incubated overnight. After washing with MEM-vitamin, 100 µL of two-fold serially diluted samples (MGO or NA inhibitors in MEM-vitamin) were added. Cells were subsequently infected with 100 µL of influenza virus B solution (B/Lee/40, B/Brisbane/60/2008, B2014/1, B2014/4, B2014/6, B2014/7 or B2014/8 in MEM-vitamin) equivalent to 100 $TCID_{50}$. The culture plates were incubated at 37°C in an atmosphere of 5% CO_2 for three days. Basically, infected cells were detached from the culture plate and viable cells still remained on the culture plate. Although CV staining could not distinguish between viable and dead cells, we performed CV staining after a 3-day incubation to assure that infected cells will be detached from culture plates. Moreover, we first evaluated the cytotoxicity of MGO using the WST-1 assay and CV staining and found that 50% cytotoxicity concentration (CC_{50}) values evaluated by the WST-1 assay and CV staining assay were similar (1.6 ± 0.4 mM *vs*. 1.4 ± 0.4 mM, respectively) (*21*). We decided to use CV staining for further evaluation. After incubation, the culture medium was removed, and 200 µL of 70% ethanol was added for 5 min and then removed. Cells were stained with 200 µL of 0.5% crystal violet (CV), purchased from Wako Pure Chemical Industries, Ltd., in water for 5 min. After washing with water and air drying, absorbance was measured at 560 nm using an Infinite M200 Tecan plate reader (Wako Pure Chemical Industries, Ltd.). The 50% virus inhibitory concentration (IC_{50}) of each sample was calculated from dose-response curves, and the percentage of viable MDCK cells was plotted.

The criteria recommended by the World Health Organization (WHO) Antiviral Working Group for data interpretation of resistant phenotypes are related to fold changes in IC_{50} values compared with those of the susceptible viruses, and the criteria for influenza B viruses are different from that of influenza A viruses. Influenza B was described as having 'normal inhibition' (< five-fold higher IC_{50} than that of the susceptible reference virus), 'reduced inhibition, RI' (five-50-fold higher IC_{50} than that of the susceptible reference virus) or 'highly reduced inhibition, HRI' (> 50-fold higher IC_{50} than that of the susceptible reference virus) (*22*).

3. Results

3.1. *Susceptibility to NAIs of influenza B viruses*

We evaluated the antiviral activities against influenza B viruses of commercial NAIs to determine the susceptibility to each drug of several strains of influenza B viruses. As reported in several studies, influenza B viruses exhibited the least susceptibility to NAIs (*14,15,23*). Our results also revealed that the mean IC_{50} of oseltamivir against laboratory B strains (B/Lee/40 and B/Brisbane/60/2008) increased approximately ten-fold relative to the IC_{50} values against laboratory A strains (A/WSN/33, A/PR/8, A/HK), as shown in Figures 2A and 2B. On the other hand, the efficacy of oseltamivir was not significantly different among the drug-sensitive clinical strains of A and B influenza viruses. For example, the IC_{50} value of oseltamivir against drug-sensitive clinical influenza A viruses (A/2009/no.6 and A/2009/no.33) ranged from 36-39 µM, which was not much different from that of oseltamivir against drug-sensitive clinical influenza B viruses (B/2014/6 and B/2014/8), which ranged from 11-33 µM (Figures 2C, 2D and Table 1). The IC_{50} of oseltamivir against drug-resistant influenza B viruses was also >500 µM. Resistance to zanamivir in influenza B was not detected in either laboratory strains or clinical strains (Figures 3A and 3C); however, the susceptibilities of some clinically isolated influenza B viruses decreased relative to those of the influenza A viruses (Figures 3B and 3D) (Table 1). Interestingly, emergence of influenza B viruses with resistance to oseltamivir (Figure 2C) tended also to be resistant to laninamivir (Figure 4C) and peramivir (Figure 5C) (Table 1). Resistance of oseltamivir has also been shown to be cross-resistant to peramivir (*24*). We also demonstrated correlations between the drug resistance of oseltamivir and peramivir not just for influenza A viruses (Figures 2D and 5D) but also for influenza B viruses (Figures 2C and 5C). The susceptibility to peramivir of influenza B viruses dramatically decreased, as shown by the 20-fold and almost 70-fold increases in the mean IC_{50} values for laboratory strains and drug-sensitive clinical strains, respectively (Table 1). On the basis of the criteria recommended by WHO, influenza B was determined to show reduced inhibition and highly reduced inhibition to peramivir for laboratory strains and drug-sensitive clinical strains, respectively.

3.2. *Antiviral activity of MGO against influenza virus B strains*

In our previous study we evaluated the cytotoxicity of MGO and activity of MGO against influenza A viruses by using MDCK cells. We first evaluated the cytotoxicity of MGO using MDCK cells and determined a CC_{50} value of 1.4 ± 0.4 mM. Our study reported that MGO obviously suppressed influenza A virus replication in a strain-independent manner (*21*). However, the antiviral activity of MGO against influenza B types has not yet been evaluated. We first evaluated the inhibitory effect of MGO against influenza B viruses by using the same experimental

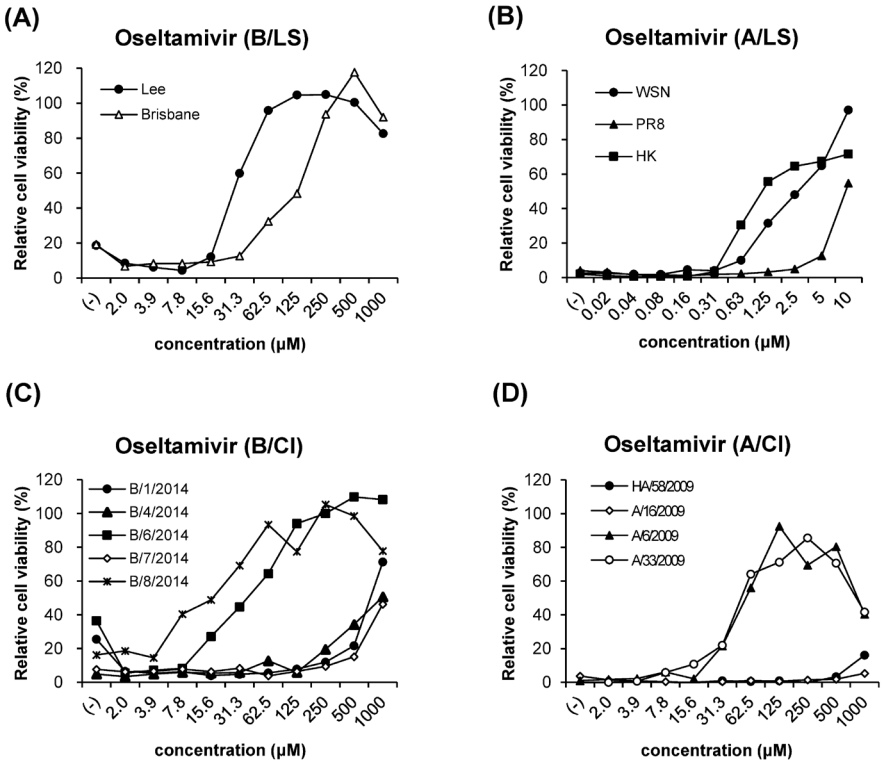

Figure 2. Anti-influenza viral activity of oseltamivir. Evaluation of the anti-influenza viral activity of oseltamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of oseltamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of oseltamivir (μM). **(A)**, **(B)**, **(C)**, and **(D)** show the susceptibilities of oseltamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

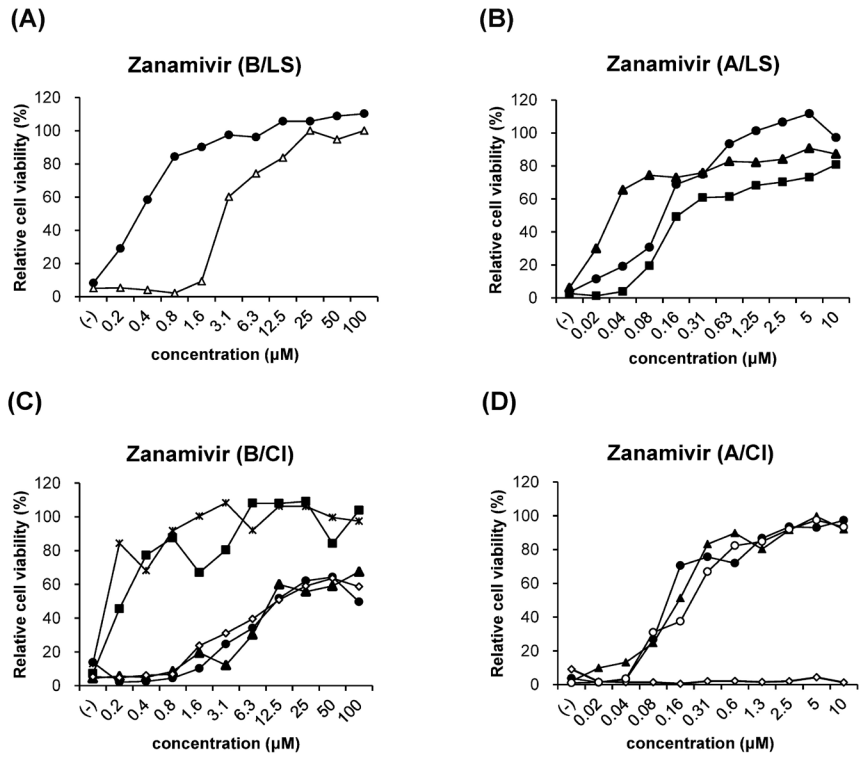

Figure 3. Anti-influenza viral activity of zanamivir. Evaluation of the anti-influenza viral activity of zanamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of zanamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of zanamivir (μM). **(A)**, **(B)**, **(C)**, and **(D)** show the susceptibilities of zanamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

Table 1. Efficacy of NAIs and MGO against various strains of IFV*

Compound	Virus strain		[a] IC_{50} (μM)	Ave. (relative)		Virus strain		IC_{50} (μM)	Ave. ([b] relative ratio)	
Oseltamivir	B/Lee/40	[c] LS	23 ± 3.5	49 (11.7)	S	A/WSN (H1N1)	LS	2.5 ± 0.5	4.2 (1.0)	S
	B/Brisbane	LS	75 ± 48		S	A/PR/8 (H1N1)	LS	9.4 ± 0.9		S
	B/2014/6	[d] CI	33 ± 6.5	22 (5.2)	S	A/HK (H3N2)	LS	0.7 ± 0.03		S
	B/2014/8	CI	11		S	A/no.6 (2009)	CI	36 ± 28	37.5 (8.9)	S
	B/2014/1	CI	> 810 ± 270	> 926.7 (220.6)	R	A/no.33 (2009)	CI	39 ± 16		S
	B/2014/4	CI	970		R	A/HA-58 (2009)	CI	870	> 935 (222.6)	R
	B/2014/7	CI	> 1000		R	A/no.16 (2009)	CI	> 1000		R
Zanamivir	B/Lee/40	LS	0.32 ± 0.06	1.51 (10.1)	S	A/WSN (H1N1)	LS	0.11 ± 0.02	0.15 (1.0)	S
	B/Brisbane	LS	2.7 ± 5.2		S	A/PR/8 (H1N1)	LS	0. 24 ± 0		S
	B/2014/6	CI	0.2	6.2 (41.3)	S	A/HK (H3N2)	LS	0. 11 ± 0.03		S
	B/2014/8	CI	< 0.2		S	A/no.6 (2009)	CI	1.7 ± 0.8	1.34 (8.9)	S
	B/2014/1	CI	11.7		S	A/no.33 (2009)	CI	2.2 ± 0.5		S
	B/2014/4	CI	9.9		S	A/HA-58 (2009)	CI	0.11 ± 0.02		S
	B/2014/7	CI	9.0		S	A/no.16 (2009)	CI	> 100	> 100 (666.7)	R
Laninamivir	B/Lee/40	LS	4.0 ± 0.21	13.5 (7.0)	S	A/WSN (H1N1)	LS	1.2 ± 0.3	1.93 (1.0)	S
	B/Brisbane	LS	23 ± 3.3		S	A/PR/8 (H1N1)	LS	1.6 ± 0.1		S
	B/2014/6	CI	12 ± 4.5	14.5 (7.5)	S	A/HK (H3N2)	LS	3.0 ± 0.8		S
	B/2014/8	CI	17		S	A/no.6 (2009)	CI	48 ±13	28.73 (14.9)	S
	B/2014/1	CI	> 500	> 500 (259.1)	R	A/no.33 (2009)	CI	35 ± 5.3		S
	B/2014/4	CI	> 500		R	A/HA-58 (2009)	CI	3.2 ± 0.3		S
	B/2014/7	CI	> 500		R	A/no.16 (2009)	CI	> 500	> 500 (259.1)	R
Peramivir	B/Lee/40	LS	0.2 ± 0.06	0.52 (20)	S	A/WSN (H1N1)	LS	0.011 ± 0	0.026 (1.0)	S
	B/Brisbane	LS	0.84 ± 0.04		S	A/PR/8 (H1N1)	LS	0.061 ± 0		S
	B/2014/6	CI	2.8 ± 0.1	1.75 (67.3)	S	A/HK (H3N2)	LS	< 0.005		S
	B/2014/8	CI	0.7		S	A/no.6 (2009)	CI	0.12 ± 0.03	0.095 (3.6)	S
	B/2014/1	CI	17 ± 3.5	> 22.3 (859)	R	A/no.33 (2009)	CI	< 0.07 ± 0.03		S
	B/2014/4	CI	> 25		R	A/HA-58 (2009)	CI	> 2.5	> 13.75 (528.8)	R
	B/2014/7	CI	> 25		R	A/no.16 (2009)	CI	> 25		R
MGO	B/Lee/40	LS	39 ± 10	31 (0.09)	S	A/WSN (H1N1)	LS	240 ± 190	340 (1.0)	S
	B/Brisbane	LS	23 ± 6.9		S	A/PR/8 (H1N1)	LS	360 ± 130		S
	B/2014/6	CI	48 ± 29	89 (0.26)	S	A/HK (H3N2)	LS	420 ± 140		S
	B/2014/8	CI	140 ± 19		S	A/no.6 (2009)	CI	195 ± 79	234.5 (0.7)	S
	B/2014/1	CI	110 ± 5.7		S	A/no.33 (2009)	CI	246 ± 2		S
	B/2014/4	CI	59		S	A/HA-58 (2009)	CI	250 ± 140		S
	B/2014/7	CI	88		S	A/no.16 (2009)	CI	247 ± 3.7		S

*Influenza virus strains were grouped into 3 groups, laboratory strain, drug-sensitive clinical strain, and drug-resistant clinical strain for influenza A and B viruses. Average IC_{50} was calculated and represented in this table for each influenza group. Number in round bracket after average IC_{50} represents relative ratio. [a] IC_{50}: 50% inhibitory concentration. [b] relative ratio: The ratio average IC_{50} against each influenza group was calculated relative to average IC_{50} against influenza A laboratory strains. It was determined the differences of susceptibility of NAI against IFV in each group. [c] LS: laboratory strain. [d] CI: clinically isolated strain.

method used for evaluation of influenza A viruses to compare the anti-influenza viral activity of MGO between influenza types A and B, as shown in Figure 6. The viral cytopathic effect was suppressed in the presence of MGO in a dose-dependent manner for all influenza virus B strains, not only laboratory strains (Figure 6A), B/Lee/40 and B/Brisbane/60/2008, but also clinical strains (Figure 6C), B/2014/1, B/2014/4, B/2014/6, B/2014/7 and B/2014/8. The IC_{50} values of MGO ranged from 23-140 μM against influenza B viruses and from 195-420 μM against influenza A viruses (Table 1), which indicated greater sensitivity of the influenza B viruses than the influenza A viruses (Figures 6B and 6D). Median IC_{50} values of MGO against influenza B were 0.09-fold and 0.26-fold lower than those of influenza A viruses for laboratory strains and clinical strains, respectively.

4. Discussion

Influenza virus is a serious threat to human health. Influenza type A and B viruses share similar family characteristics, such as the negative single-strand RNA. However, there are also significant differences in epidemiology, evolutionary pattern, and host reservoir between the viruses (*25,26*). The emergence of influenza viruses with reduced susceptibility to NAIs has also been a critical issue recently, especially for influenza A and B viruses. Thus, the development of novel anti-influenza viral drugs is urgently required. Based on the finding of our recent report regarding the anti-influenza A viral activity of MGO (*21*), we hypothesised that MGO would also be effective against influenza B viruses in MDCK cells. Our present data indicate that MGO has antiviral activity against

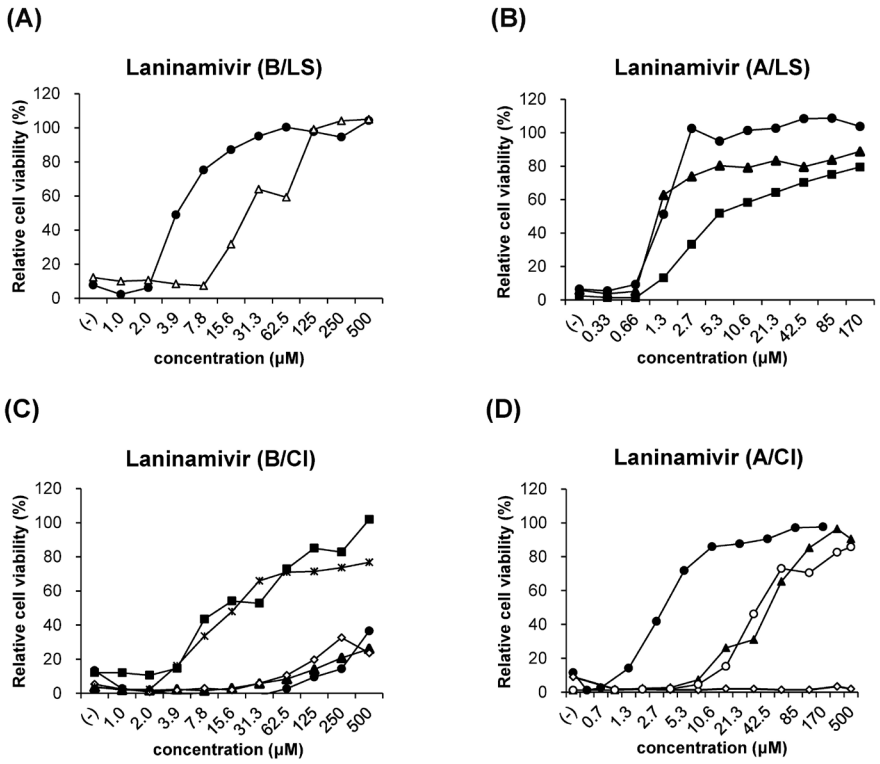

Figure 4. Anti-influenza viral activity of laninamivir. Evaluation of the anti-influenza viral activity of laninamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of laninamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of laninamivir (μM). **(A)**, **(B)**, **(C)**, and **(D)** show the susceptibilities of laninamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

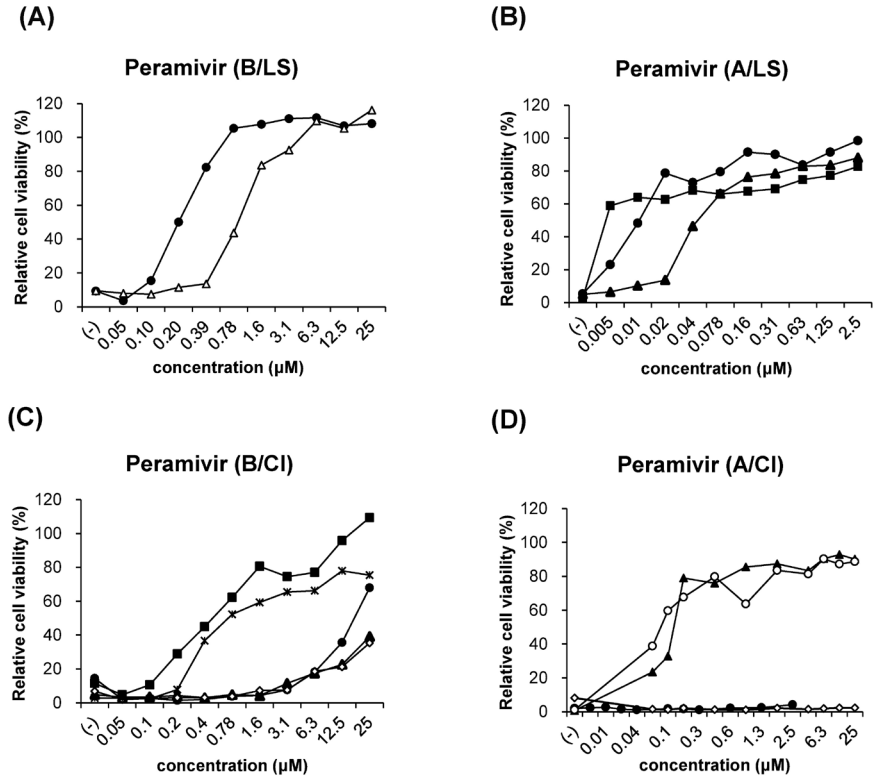

Figure 5. Anti-influenza viral activity of peramivir. Evaluation of the anti-influenza viral activity of peramivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of peramivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of peramivir (μM). **(A)**, **(B)**, **(C)**, and **(D)** show the susceptibilities of peramivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

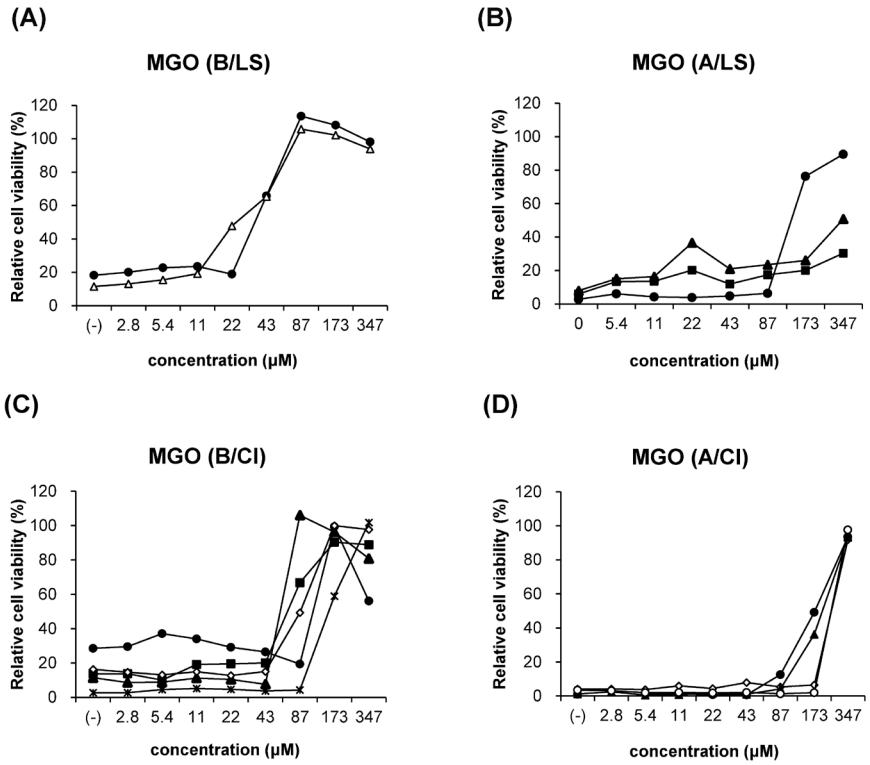

Figure 6. Anti-influenza viral activity of MGO. Evaluation of the anti-influenza viral activity of MGO was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of MGO and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of MGO (μM). **(A)**, **(B)**, **(C)**, and **(D)** show the susceptibilities of MGO to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

influenza B viruses, including influenza B virus with reduced susceptibility to NAIs (Figure 2 and Table 1). Interestingly, the susceptibility of MGO against influenza B viruses was higher than that against influenza A viruses, as suggested by the ten-fold and 3.8-fold reduced IC_{50} values for laboratory strains and clinical strains, respectively (Table 1).

Based on our finding that MGO shows anti-influenza activity in a strain-independent manner and can inhibit replication of influenza viruses with reduced susceptibility to NA inhibitors, the mechanism of MGO may not be related to interactions of HA or NA, in which mutation easily occurs. Some studies have reported that MGO showed antiviral activity against foot-and-mouse disease (*27*) and Newcastle disease virus (*28*) *via* interaction with viral RNA. The infectiousness of RNA isolated from MGO-treated virus was not infectious in subcutaneous inoculation of mice (*27*). The influenza virus polymerase does not possess a proof-reading function, so the virus rapidly adapts to certain selection pressures, thereby generating resistant viruses, especially if a viral protein is a drug target, such as an NAI. Because the extremely high mutation rate of influenza virus leads to the emergence of new virus strains that create a crucial problem for development of antiviral agents, the cellular cofactors that are necessary during influenza virus infection could be new targets for drug development. Several studies have reported that during infection, influenza viruses activate the Raf/MEK/ERK-cascade and the transcription factor nuclear factor kappa B (NF-κB) (*29-32*). The Raf/MEK/ERK-cascade is activated by influenza virus to support viral propagation, while inhibition of this cascade impairs function of the nuclear export protein, which results in accumulation of ribonucleoprotein complexes (RNPs) in the nucleus (*33,34*). Like the Raf/MEK/ERK-cascade, NF-κB is activated by influenza infection, then induces caspases, which subsequently supports the replication of influenza virus by enhancing RNP export (*30,35*). Molecules interfering with the NF-κB pathway, such as acetylsalicylic acid (ASA) (*35*), have been reported to have antiviral activity (*31*). Inhibition of cellular signalling of antiviral activity may be a novel function of new anti-influenza agents that target host-cell functions. Most importantly, no resistant virus variants have been shown to emerge in the presence of cellular pathway inhibitors, which suggests that influenza viruses cannot easily adapt to missing crucial cellular functions (*34,35*).

MGO has been reported to suppress tumor necrosis factor-α-induced NF-κB activation by inhibition of NF-κB DNA-binding and NF-κB-dependent reporter gene expression in a concentration-dependent manner (*36*), which is consistent with our data showing that MGO

can inhibit influenza replication in a dose-dependent manner. Similar to ASA, which blocks influenza virus by inhibiting NF-κB activity, MGO may inhibit influenza virus replication by interfering with NF-κB activation. Therefore, MGO can suppress influenza viral replication in a strain-independent manner.

The emergence of NAI-resistant influenza B viruses is a major public health concern worldwide. The emergence of NAI-resistant influenza B virus information is not well-understood, and concerns are often underestimated relative to those of influenza A viruses, although both viruses are regarded to cause significant disease burdens and to a similar degree. In Japan, the rate of NAIs used for clinical treatment have been found to be much higher than anywhere else in the world, and the use of NAIs has caused the spread of influenza B viruses with reduced susceptibility to NA inhibitors (*16*). Our results show that influenza A viruses are more susceptible to NAIs than influenza B viruses. The patterns observed for drug susceptibility were similar to those previous published (*37*). One possible explanation is that the binding affinities of HA protein of influenza B viruses and the sialic acid moiety are weaker than those of influenza A viruses (*37,38*). The problem of wild type influenza B viruses already having reduced susceptibility to NAIs relative to that of influenza A viruses is a concern because any further increase in IC_{50} values due to mutations may induce complete loss of drug effectiveness in influenza B treatment or prophylaxis. Although the similarity of the amino acid composition of NAs between influenza A and B viruses is only 30% (*39*), the 19 amino acids at the catalytic site are highly conserved among all known influenza A and B NAs (*40*). Several clinical studies have reported that the locations of NA mutations differs among the NAIs used (*26*), and the locations of NA substitutions confer different levels of resistance among the NAIs used (*37,41,42*).

NA mutations of oseltamivir were observed at a higher rate than the rate for NA mutations of zanamivir. This phenomenon is because zanamivir is more similar to Neu5ac than is oseltamivir, so the binding of zanamivir to the NA active site is similar to natural substrate binding. Moreover, the rate of use of zanamivir is clinically lower than that of oseltamivir (*43*). Laninamivir is a long-acting derivative of zanamivir, which is administered as a single inhaled dose. The advantages of laninamivir are that it not only resides in the lung for many days (*44*) but also has slower dissociation than that of other NA inhibitors (*45*). Mutations at the location affecting the laninamivir dissociation rate can confer a dramatic resistance to laninamivir (*46*). Some studies have reported that mutations conferring zanamivir resistance also induce resistance to laninamivir with the loss of slow binding and/or faster dissociation (*46*), also relevant to our results (Table 1). Although our results do not show any resistance of influenza B viruses to zanamivir, the median IC_{50} of zanamivir against clinical strains resistant to other NA inhibitors (B/2014/1, B/2014/4, B/2014/7) was approximately 50-fold greater than those of clinical strains sensitive to other NA inhibitors (B/2014/6, B/2014/8). On the basis of the criteria recommended by WHO, B/2014/1, B/2014/4, and B/2014/7 were classified as showing highly reduced susceptibility to inhibition by zanamivir and all NAIs. Peramivir contains a guanidino group, as does zanamivir, and a hydrophobic group, as does oseltamivir; consequently, mutations affecting the activities of oseltamivir and zanamivir can also confer resistance to peramivir (*47*), and is supported by our results.

Although influenza A and B viruses belong to the family of Orthomyxoviridae, they possess distinct characteristics that are grouped into different types. Currently, several researchers are interested in influenza virus–host interactions. A difference in apoptosis profiles between influenza A and B strains has been reported recently (*48*). Influenza B viruses induce apoptosis earlier in infection, whereas influenza A viruses delay induction of apoptosis. Moreover, the transcription mechanisms of influenza A and B viruses enabled influenza B polymerase to recognize the cap structure in a manner different from that of influenza A polymerase, and the growth of influenza B viruses was more sensitive to the amount of cellular mRNA than was growth of influenza A viruses (*49*). These phenomena may explain our finding that MGO was more sensitive to influenza B viruses than to influenza A viruses and had a synergistic effect with NAIs only on influenza A viruses and not on influenza B viruses (data not shown).

In conclusion, our results show that MGO has potent inhibitory activity against influenza viruses A and B, including influenza viruses with reduced susceptibility to NAIs. Although influenza B viruses have been reported to be less sensitive to NA inhibitors than influenza A viruses, MGO shows higher sensitivity to influenza B, without observed resistance. Therefore, MGO has high potential as a universal anti-influenza agent, including potential for activity against resistant pandemic influenza viruses. A study to elucidate the mechanism of MGO and to determine whether it interacts with cellular mechanisms is in progress.

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Original Article

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Biophysical characterization of a model antibody drug conjugate

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Summary **Antibody drug conjugates (ADC) are important next-generation biopharmaceuticals and thus require stringent structure characterization as is the case for monoclonal antibodies. We have tested several biophysical techniques, *i.e.*, circular dichroism, analytical ultracentrifugation, differential scanning calorimetry and fluorescence spectroscopy, to characterize a fluorescein-labeled monoclonal antibody as a model ADC. These techniques indicated possible small structure and stability changes by the conjugation, while largely retaining the tertiary structure of the antibody, consistent with unaltered biological activities. Thus, the above biophysical techniques are effective at detecting changes in the structural properties of ADC.**

Keywords: Circular dichroism, sedimentation velocity, calorimetry, antibody drug conjugate, biophysical characterization

1. Introduction

Targeted delivery of anti-cancer drugs and radioactive isotopes using cancer-specific antibodies, lipids and other compounds is currently being studied extensively as next generation biopharmaceuticals or diagnostic reagents (*1-8*). When antibodies and drugs are combined to make antibody-drug conjugates (ADC), also known as armed antibodies, the conjugation of what is often a hydrophobic drug to the antibody has the potential to alter the chemical and physical properties of the ADC. Pharmaceutical proteins, including antibodies, require extensive characterization of their structural properties, such as aggregation, conformation and stability (*9-12*). Such characterization is also required for ADC.

A number of biophysical techniques are used to characterize pharmaceutical proteins. Circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) are used to characterize protein conformation. Protein aggregation is commonly characterized by dynamic light scattering (DLS), static light scattering combined with size exclusion chromatography (SEC-MALS) and analytical ultracentrifugation (AUC). Differential scanning calorimetry (DSC) is used to characterize the thermal stability of proteins. It would be of great interest to see if these techniques can be successfully applied to characterize ADCs, as the conjugated drugs may interfere with the optical and hydrodynamic properties of ADCs (*13*). Here, we have initiated a study on the biophysical characterization of a model ADC. For this model ADC, we have decided to use a fluorescent probe, fluorescein isothiocyantae (FITC), as an alternative compound to anti-cancer drugs. FITC has aromatic rings, similar to aromatic hydrophobic drugs and hence its conjugation with antibodies will likely challenge the structural features of the antibody in a manner similar to a conjugated drug. We have compared the intact (*i.e.* the non-labeled) and the FITC-labeled monoclonal antibody (mAb) using CD, fluorescence, DSC and AUC. Isoelectric focusing (IEF) was used to confirm FITC conjugation, which was also determined by UV and visible absorbance spectroscopy.

2. Materials and Methods

2.1. *Antibody preparation*

A rabbit IgG monoclonal antibody was generated against a phosphorylated peptide. Conditioned medium (CM) expressing the IgG in Chinese hamster ovary cells was subjected to Protein-A chromatography. After

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loading the CM, the column was washed with 1 M arginine, 20 mM phosphate, pH 6.0 followed by elution of the bound IgG using Ajinomoto's Protein-A elution buffer (0.7 M arginine, 20 mM acetate, pH 4.1) (*14,15*). The eluted IgG solution was dialyzed against 10 mM sodium bicarbonate, pH 8.8.

2.2. *FITC labeling*

The above antibody solution (10 mL) was adjusted to 1 mg/mL protein. FITC (10-fold molar excess based on the molecular mass of 140,000 for the IgG) dissolved in 0.1 mL DMSO was added to the above antibody solution and incubated at room temperature for 15 hours. Unconjugated free FITC was removed by cation exchange Capto MMC mixed-mode chromatography in 20 mM phosphate at pH 6.5 and anion exchange Capto adhere mixed-mode chromatography in 50 mM Tris-HCl at pH 8.6. In both cases, FITC-labeled IgG bound to the column, while free negatively charged FITC flowed through the Capto MMC column. The bound FITC-IgG was eluted from the Capto MMC column with an arginine gradient from 0 to 1 M at pH 6.5 and from the Capto adhere column with an arginine gradient from 0 to 1.5 M at pH 8.6. The eluted FITC-IgG solution was dialyzed against 10 mM phosphate, pH 6.5. The intact protein was also dialyzed into the identical buffer.

2.3. *CD spectroscopy*

CD measurements were done using 0.416 mg/mL antibody solution (both FITC-IgG and intact IgG) in 10 mM phosphate, pH 6.5. Near and far UV CD spectra were determined at room temperature using a Jasco J-715 spectropolarimeter using 1 and 0.5 cm path-length cells. The spectra of free FITC were also determined using a solution of FITC that was equivalent to the concentration present in an FITC-IgG1 sample at 0.416 mg/mL. After subtracting the buffer spectrum, the CD spectra were converted to the mean residue ellipticity using the path-length of the cell, the protein concentration and the mean residue weight of 108 g/mol.

2.4. *Isoelectric focusing*

IEF experiments were done using 3-10 IEF gels, anode and cathode tank buffers, IEF sample buffer and IEF markers all from Life Technology. Electrophoresis was run by sequentially increasing the voltage from 100, 200 to 300 at 1 hour intervals. The gel was stained with Coomassie blue SimplyBlue SafeStain, also from Life Technology.

2.5. *Analytical ultracentrifugation sedimentation velocity*

Sedimentation velocity experiments were carried out using a Beckman XLI analytical ultracentrifuge. The parent IgG and FITC-IgG molecules were dialyzed against 10 mM phosphate, pH 6.5. The protein was diluted to 0.0.42 mg/mL using the dialysate and loaded (~450 µL) into 2-channel charcoal-epon centerpieces with a 12 mm optical pathlength using the dialysate as the reference solution. Data were collected at 280 nm, every 0.003 cm with no averaging in the continuous scan mode. The raw data were analyzed using the SEDFIT program (*16*) to obtain the sedimentation coefficient distribution.

2.6. *Differential scanning calorimetry*

The sample and reference cells of a TA Instruments Nano DSC were loaded using the autosampler with ~ 0.3 mL of sample and formulation buffer, respectively. The instrument was programmed to scan from 5 to 105°, at a rate of 60°C/hr, with a 5 second data averaging period. Several buffer *vs*. buffer scans were recorded throughout the experiment sequence to obtain a baseline scan to subtract from the experimental data and to ensure the sample and reference cells were adequately cleaned over the course of the experiment. Upon completion of a scan, the machine was programmed to clean the cells with a 5% Contrad solution, followed by an exhaustive water wash, and then the cells were reloaded with the next sample/buffer pair. The raw data were processed using NanoAnalyze version 3.1.2. A buffer-buffer scan was subtracted from each sample-buffer scan, and the baseline was calculated and processed using the NanoAnalyze software according to the manufacturer's instructions. The C_p profiles were normalized to protein concentration (expressed as kcal/mol/°C).

2.7. *Fluorescence spectroscopy*

The fluorescence emission spectra were collected using a Horriba Jobin Yvon FluoroMax-4 specrofluorometer. The excitation wavelength was set to 280 nm, and the emission spectra were collected at 90° from the excitation light source, from 295 to 500 nm, with an integration time of 0.2 s. The excitation and emission slits were set to 2 and 4 mm, respectively. The excitation and emission monochromators were calibrated according to the manufacturer's instructions using the water Raman peak. To obtain a true emission spectrum (*i.e*. independent of the instrument used to collect it), the spectra were corrected for instrument dependent factors according to the manufacturer's instructions. Spectra were collected in a 4 mL quartz cuvette with a 1 cm pathlength. The experiments were performed at 25°C in a thermostatted cuvette holder fitted with a circulating water bath. A buffer background spectrum was collected and subtracted from each sample spectra to correct for small amounts of fluorescence/scattering

from the buffer and to subtract the water Raman peak from the sample spectra. Spectra were collected within the manufacturer's recommended linear range of the instrument (1-2 million counts per second, CPS). Each sample was diluted to 50 µg/mL directly into the cuvette and was mixed by gently pipetting with a 1 mL pipetman.

2.8. *Bioassay*

A 96-well titer plate was coated with either non-phosphorylated or phosphorylated peptide in the presence of bovine serum albumin (BSA), washed with phosphate-buffered saline (PBS) and blocked with 1% BSA in PBS. The intact IgG1 and FITC-IgG were diluted to 2.5 µg/mL with PBS containing 1% BSA and then serially diluted 4-fold with the same buffer. These diluted samples were added to the wells and incubated at 37°C for 1 hour. The wells were then washed with PBS, and then HRP-conjugated goat anti-rabbit antibody was added for detection. The cells were washed with PBS again and developed with ultra TMB-ELISA substrate. Reaction was stopped after 5 min with 2 N H_2SO_4. The plate was read at 450 nm.

3. Results and Discussion

It is expected that the number of conjugated drugs per antibody molecule determines not only pharmacological efficacy but also spectroscopic and hydrodynamic properties of the ADCs. Thus, focus has been given to determine the ratio of the drug to the antibody and its heterogeneity using various chromatographic techniques (*13*). Here, we show below structure characterizations of ADCs using non-chromatographic techniques.

3.1. *Isoelectric focusing*

FITC reacts with amino groups of the antibody and hence reduces one positive charge per labeling. FITC has a negative charge which adds one negative charge per labeling. Therefore, in total, labeling one amino group results in the addition of 2 net negative charges, which should decrease the isoelectric point of the IgG. Figure 1 shows IEF analysis using a pH 3-10 gel system for the intact IgG1. Several protein bands around pH 8.3 were observed in part due to heterogeneous glycosylation. Figure 1 also shows the distribution of pI isoforms after FITC labeling. The number of stained bands greatly increased, indicating that the labeling resulted in increasing heterogeneity as expected from random labeling. The bands distributed around pH 7.0, indicating that the pI shifted to a lower pH as expected from increased net negative charges. The large shift in pI clearly demonstrates that the IgG has been labeled by FITC.

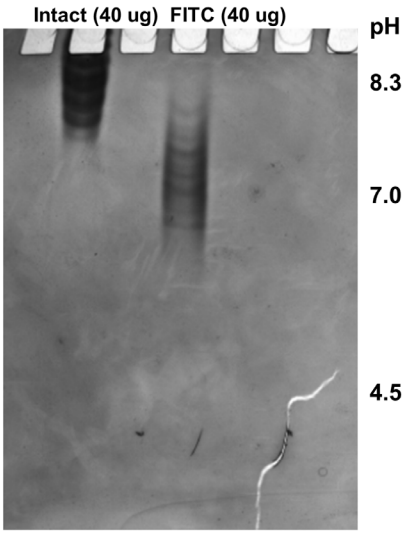

Figure 1. Isoelectric focusing. pH 3-10 gel was loaded 40 µg each of intact IgG and FITC-IgG.

3.2. *Labeling ratio*

FITC has absorbance in the visible region with a maximum at 491 nm and in the UV region (*e.g.*, at 280 nm) where protein absorbs. The ratio of UV absorbance at 280 nm to the value at 491 nm for FITC was determined to be 0.52 from the absorbance spectrum of free FITC. The UV absorbance spectrum of FITC-IgG showed the absorbance at 491 nm as 0.35 and at 280 nm as 0.76. Using the above ratio, the contribution of FITC absorbance at 280 nm was calculated to be 0.18, indicating the UV absorbance at 280 nm from the protein portion of FITC-IgG to be 0.58. Using the extinction coefficient of 1.4, the protein concentration was calculated to be 0.416 mg/mL. From the absorbance of 0.35 at 491 nm, the concentration of FITC in the FITC-IgG solution was calculated to be 3.13×10^{-3} mg/mL based on the absorbance of free FITC. Converting these weight concentrations to molar concentrations, the labeling ratio was determined to be 2.8 mol FITC per mol protein. Thus, labeling adds on average 5.6 net negative charges and 2.8 molecules of fluorescein per protein molecule. Such addition should also increase hydrophobic binding to both Capto MMC and Capto adhere (that were used to remove free FITC) and should decrease electrostatic binding to Capto MMC but increase electrostatic binding to Capto adhere. Since labeling is expected to increase both hydrophobic and electrostatic binding, Capto adhere may be utilized to fractionate FITC-IgG with different degrees of labeling. Although the Capto adhere chromatography used above did not show separation, a more optimal elution condition may be developed for fractionation of labeled isoforms. It is expected that higher labeling has stronger binding to Capto adhere column through enhanced hydrophobic and electrostatic interactions (when amino groups are used for conjugation).

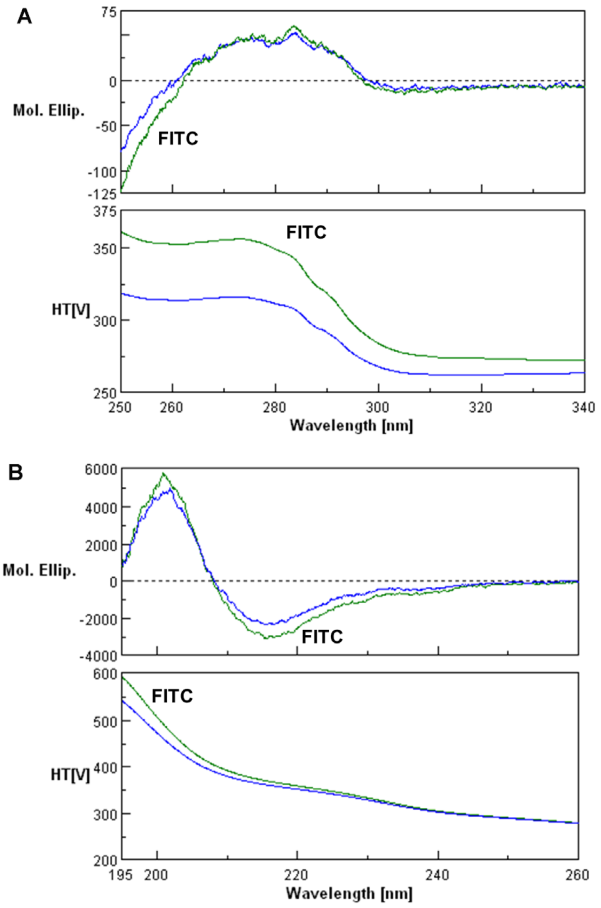

Figure 2. Near (A) and far (B) UV CD spectra. Intact IgG, blue; FITC-IgG, green.

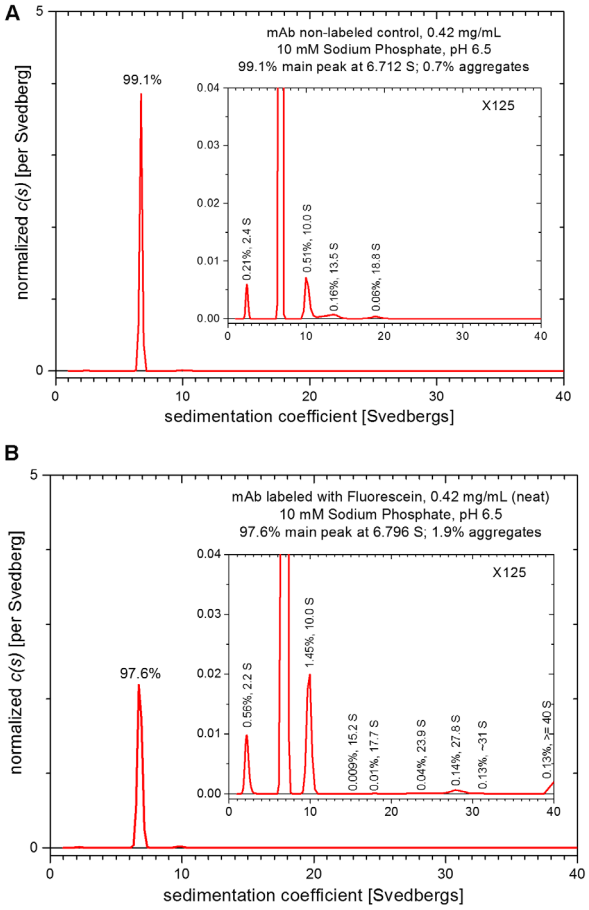

Figure 3. Sedimentation velocity analysis of the intact and FITC-IgG antibodies. (A) Intact IgG, **(B)** FITC-IgG1. The inset shows a magnified view so that the minor peaks can be seen.

3.3. *Circular dichroism*

CD measurements were carried out at 0.416 mg/mL protein for both intact and FITC-IgG in 10 mM phosphate, pH 6.5. Figure 2A shows the near UV CD spectra (upper panel) and HT[V] spectra (lower panel) of intact IgG1 (blue) and FITC-IgG (green). The HT[V] signals closely follow the absorbance properties of the samples and are stronger for FITC-IgG1 (green), despite an identical protein concentration, reflecting that it has contribution from FITC absorbance. This difference in UV absorbance was used to determine the FITC and protein concentration of the conjugated molecule. The CD spectra, expressed as mean residue ellipticity, were nearly identical above 260 nm, indicating that FITC labeling does not alter the tertiary structure of the protein nor generate new CD signals: note that free FITC itself has no CD in this wavelength region (data not shown). It is possible that FITC conjugation to the antibody generates CD signals. Figure 2B shows the far UV CD spectra of the intact (blue) and FITC-IgG (green) in the same buffer. The HT[V] signals are slightly stronger for FITC-IgG1 in this region, consistent with the fact that free FITC has weak absorbance (data not shown). There is no far UV CD as well with free FITC (data not shown). Both spectra have a minimum at 217 nm and a maximum at 202 nm, characteristic of antibody structure. However, the CD intensity appears to be significantly stronger for the FITC-IgG, suggesting a small effect of labeling on the secondary structure of IgG.

As many cytotoxic drugs have UV absorbance that can interfere with optical properties of ADCs (*13*), this study demonstrates that care should be exercised to determine accurate protein concentration for CD analysis and then CD can be used to assess the effects of drug conjugation on the secondary and tertiary structures of the ADCs.

3.4. *Sedimentation velocity*

Figure 3 shows sedimentation velocity experiments that were carried out at 0.42 mg/mL for both the labeled FITC-IgG (green) and unlabeled IgG (blue), at 40,000 RPM and 20°C in 10 mM phosphate, pH 6.5. The raw data were analyzed by the c(s) method to obtain the size distribution. Each distribution was normalized so that the total area under the curve is

equal to 1. The FITC-IgG monomer sediments at 6.796 S while the unlabeled (intact) IgG monomer sediments at 6.712 S. The expected intra-run variability for this experiment is ± 0.006 S; thus, we are able to detect a small increase in the sedimentation coefficient upon labeling IgG with FITC. We expect the sedimentation coefficient to increase slightly since the molar mass of the labeled compound is predicted to increase by about 0.7%. However, this technique is also sensitive to changes in shape, so it is not clear from this experiment alone if this increase is due to the increase in mass or if it is due to a slight compaction of the tertiary structure, resulting is a slightly faster sedimenting molecule. Note that the near UV CD has shown unaltered tertiary structure by FITC labeling, suggesting that the slight increase in sedimentation coefficient may in fact be due to the increased molar mass of the conjugated mAb.

The unlabeled mAb control is highly homogeneous, giving 99.1% main peak. Three peaks are detected that sediment faster than the main peak, which account for 0.7% of the total sedimenting absorbance. Presumably these peaks reflect antibody aggregates and not high molecular weight impurities. A single peak is detected that sediments slower than the main peak, at 2.4 S (0.21%). The aggregate content of FITC-IgG increases to 1.9%, and many additional peaks are detected. The total aggregate content for this sample is close to the expected LOQ for this technique (which has been estimated at 1-2% for dimers of antibodies), indicating it is not possible to determine if the labeled and unlabeled antibodies truly display different aggregate levels from this single experiment. Furthermore, note that the very minor peaks observed for FITC-IgG are almost certainly pushing the limit of detection for this technique. Nevertheless, it should be emphasized that sedimentation velocity is a powerful technique to see the effects of drug conjugation on aggregation of the ADCs.

Both the labeled and unlabeled mAbs show a peak at 10.0 S, which is sedimenting about 1.5 times faster than the respective main peaks. This ratio suggests that the 10.0 S corresponds to an antibody dimer, however, without investigating the effect of concentration on the positions and relative amounts of these observed peaks, we cannot be certain if this peak corresponds to an irreversible or reversible aggregate of the main peak material.

3.5. *Differential scanning calorimetry*

Figure 4 shows the DSC data collected for both the labeled and unlabeled mAb, carried out at a loading concentration of 0.42 mg/mL. The heat capacity profile for the unlabeled mAb looks quite similar to what has been seen in the literature for the IgG subclass (*17*). The shoulder at about 80°C corresponds to the melting

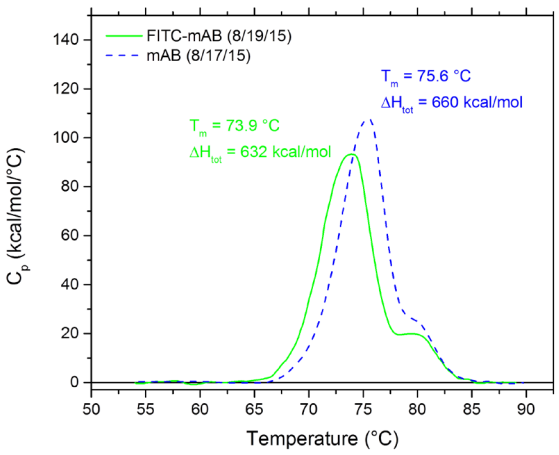

Figure 4. Differential scanning calorimetry analysis of the intact and FITC-IgG antibodies. The green trace corresponds to the FITC-IgG antibody, while the blue trace corresponds to the intact IgG antibody. The T_m values that are annotated on the graph correspond to the temperature at the maximum C_p value for the main peak. The total unfolding enthalpies (ΔH_{tot}) were calculated by numerical integration of the entire C_p curve.

of the C_{H^3} region within the Fc portion of the mAb, while the rather broad peak at 75.6°C corresponds to the F_{AB} portion of the mAb. The C_{H^2} region, usually seen around 70°C or so, is not obvious under these conditions, but it must be emphasized that separation of the C_{H^3} profile from the F_{AB} profile is often not observed, and such separation strongly depends on the solution variables, especially pH.

The FITC-IgG shows a strong decrease of 1.7°C in the apparent T_m of the presumed FAB region, from 75.6°C (non-labeled) to 73.9°C (labeled), with no apparent difference in the C_{H^3} region. This shows that the labeling of the mAb with a small, hydrophobic molecule (that only accounts for about 0.7% of the total mass) decreases the thermal stability of the molecule, and is easily detectable by DSC. While the shift in the apparent Tm is well above the expected variability for this technique (about ± 0.1°C), the apparent decrease in the total unfolding enthalpy upon labeling (4.2%) is not. The total unfolding enthalpy is dependent upon accurate knowledge of the loading concentration, which in this case carries more uncertainty than normal since it is not entirely clear how FITC affects the extinction coefficient of the protein.

3.6. *Fluorescence spectroscopy*

Figure 5 shows an overlay of fluorescence emission spectra collected for the non-labeled and the FITC-IgG mAbs. This experiment was conducted using an excitation wavelength of 280 nm. The peak emission wavelength was observed at 329 nm for both mAbs. This value shows that the tryptophan residues are largely buried and that labeling the mAb with FITC

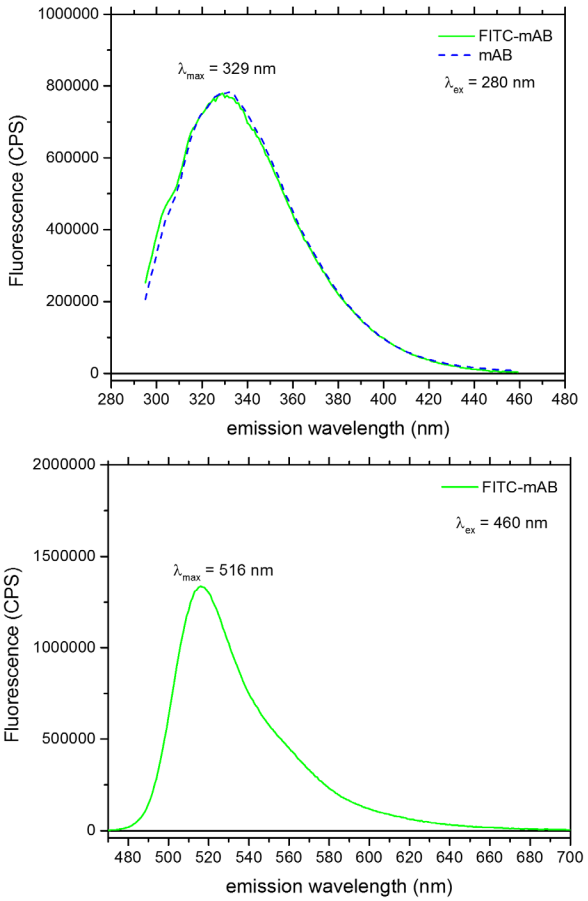

Figure 5. Fluorescence spectroscopy analysis of the intact and FITC-IgG antibodies. The green trace corresponds to the FITC-IgG antibody, while the blue trace corresponds to the intact IgG antibody. The excitation wavelength was 280 nm.

does not significantly alter the environment of these tryptophan residues.

3.7. *Binding activity*

The rabbit IgG was developed to bind to a phosphorylated peptide. Binding activity and specificity were compared for the intact and labeled IgG. Figure 6 shows dose dependence of binding to a plate coated with phosphorylated and non-phosphorylated peptides. No binding of both intact IgG and FITC-IgG was observed against non-phosphorylated peptide, indicating no non-specific binding is occurring for the labeled or intact IgG molecules. Both molecules showed dose-dependent binding to phosphorylated peptide with the FITC-IgG dose curve shifted by about 3-fold to lower protein concentrations, suggesting that FITC-IgG has a slightly higher affinity for the phosphorylated peptide.

4. Conclusion

The biophysical techniques described here are routinely used to characterize pharmaceutical proteins. Characterization of the IgG antibody showed a profile

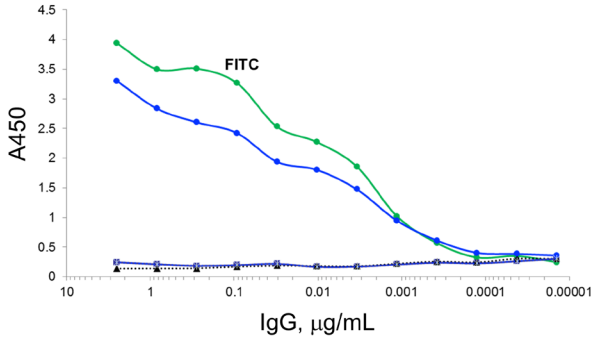

Figure 6. Bioassay of the intact and FITC-IgG antibodies.

that is characteristic of typical antibody structure in aqueous solution. Conjugation of FITC, used as a model drug in ADC, alters the secondary structure and thermal stability of the IgG, while affecting little the tertiary structure as seen by near UV CD, sedimentation velocity and fluorescence, which is consistent with unaltered biological activities. These biophysical techniques as well as separation techniques described here can be used to characterize biopharmaceutical ADC products, although it should be noted that pharmaceutical ADC contains spacer sequence between the parent antibody and the drug compound that need to be cleaved upon internalization.

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Original Article

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Daily walking decreases casual glucose level among pregnant women in the second trimester

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Summary **The objective of this study was to explore the relationship between carbohydrate metabolism and the number of steps walked daily, as evaluated by accelerometer, among Japanese women in the second trimester of pregnancy. This longitudinal study was conducted at a university hospital in Tokyo, Japan, from August 2012 to January 2013. Healthy pregnant women at 14 to 18 gestational weeks were recruited. Participants wore accelerometers on the waist for 4 weeks. Casual glucose and hemoglobin A1c (HbA1c) levels were compared between two groups based on whether participants habitually walked ≥ 6,000 steps/day or < 6,000 steps/day. Fifty-one pregnant women were included in the present study; data from 35 were analyzed. There were 22 women in the group that habitually walked ≥ 6,000 steps/day and 13 in the group habitually walking < 6,000 steps/day. Although the median serum casual glucose level at the end of the investigation was 90.0 mg/dL in the group walking < 6,000 steps/day, the level in the group walking ≥ 6000 steps/day was 83.5 mg/dL (p = 0.01). HbA1c levels were not significantly different between the two groups. Our results suggest that walking as a daily habitual physical activity is effective for controlling casual glucose levels in the second trimester of pregnancy.**

Keywords: Walking, glucose, pregnancy trimester, second

1. Introduction

Gestational diabetes mellitus (GDM) is one of the perinatal complications. This disorder of carbohydrate metabolism during the gestational period not only affects women during pregnancy, but also affects them after delivery. Previous studies have reported that women who experienced GDM developed type 2 diabetes in the future at a high rate (*1,2*). Since complete recovery is difficult once a woman develops type 2 diabetes, preventing its occurrence is of primary importance. In order to maintain a woman's health over her lifetime, it is useful to determine how GDM can be prevented.

Pregnancy itself easily induces abnormalities of carbohydrate metabolism because the placenta, which completes development in the second trimester, makes proteolytic enzymes and decomposes insulin in the mother's body. Furthermore, because Asians show the least insulin secretion of all ethnic groups (*3-5*) due to genetic factors, it is possible for them to develop disorders of carbohydrate metabolism without concomitant obesity. An unhealthy lifestyle, including poor nutrition and low physical activity, influences the expression of genes that participate in the energy metabolism process via oxidative phosphorylation of cell mitochondria and induces insulin resistance (*6*). Thus, it is necessary for pregnant Asian women to be cognizant not only of their diet but also of their physical activity. Similar to the way that many management protocols are available for preventing type 2 diabetes based on diet and physical activity, management protocols for preventing GDM must be prepared. Although many previous studies have focused on the effects of diet, physical activity has only recently become a focus. Thus, scientific findings concerning physical activity are still lacking (*7*).

Appropriate physical activity can be effective in improving carbohydrate metabolism, including glucose

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level, in the general population (*8*). However, there is currently no evidence regarding the effects of physical activity on glucose level among pregnant women. Physical activity consists of daily habitual physical activity and leisure sports. Leisure sports are defined as body movements that induce a heart rate > 140 beats/minute with a duration of over 60 minutes; that is to say, high-intensity activity. Daily habitual physical activity is defined as any form of body movement that induces energy expenditure above resting levels in daily life. Therefore, daily habitual physical activity is assessed as a low-intensity activity. Previous research indicates that daily habitual physical activity improves insulin resistance in middle-aged women (*9*). Because GDM seems to be caused by increased insulin resistance, it is likely that insulin resistance in pregnant women would improve with adequate daily habitual physical activity. The purpose of this study was to explore the relationship between daily habitual physical activity, defined as the number of steps walked, as assessed by accelerometer, and carbohydrate metabolism among pregnant Japanese women.

2. Materials and Methods

2.1. *Subjects*

This longitudinal study was conducted at a university hospital in Tokyo, Japan, from August 2012 to January 2013. One hundred and sixty-four pregnant women visited the hospital for a routine prenatal examination between 14 and 18 gestational weeks from August through December 2012, the recruiting period. Inclusion criteria were that participants should be healthy pregnant women. The exclusion criteria included pregnant women who were less than 20 years old, who could not respond to a questionnaire, who had a history of recurrent or habitual abortion, who experienced intrauterine fetal death, who had a threatened miscarriage or premature delivery, who had type 1 or type 2 diabetes, who were using steroids, who required hospitalization, and who had a pregnancy complicated by fetal disorders. Additionally, women with a casual glucose level > 100 mg/dL at ~12 gestational weeks were excluded from analyses, since they had to receive nutritional guidance due to having a high risk of developing GDM (*10*).

2.2. *Research protocol*

Daily physical activity was estimated from the number of steps taken and amount of exercise performed daily, as measured using an accelerometer (Lifecorder EX; Suzuken Co Ltd, Nagoya, Japan) (*11*), and carbohydrate metabolism was measured with casual glucose and hemoglobin A1c (HbA1c) levels.

Healthy pregnant women were recruited at 14-18 gestational weeks while they were awaiting their routine prenatal examination in the outpatient hospital room. Background information of the participants, including maternal age, gestational week, pre-pregnancy body mass index (BMI), pregnancy history, fetus number, any pregnancy complications, and laboratory biochemical data in the first trimester were obtained from patients' medical charts. Participants attached the accelerometers to the waistbands of their skirts or pants, as instructed at the time of recruitment by investigators. If participants experienced any discomfort from wearing the accelerometer on their waistbands, they wore belts that were accessories to the accelerometers and fixed the accelerometers to the belts. The accelerometers assessed their daily activity based on the number of steps taken and amount of exercise performed every day for 4 weeks; the accelerometers were removed for sleeping and bathing. They were asked not to participate in any leisure sports unless they were part of their normal habits.

After 4 weeks, the participants removed the accelerometers and completed questionnaires, which assessed dietary intake and habitual physical activities. We also collected 11 mL of blood during the second trimester for use in the present study, in addition to the blood collected during routine prenatal visits.

Because it has been reported that middle-aged women could prevent metabolic syndrome by walking at least 6,000 steps per day (*9*) and that the average number of steps taken in the second trimester by pregnant Japanese women who do not play sports was about 6,000 steps/day (*12*), participants were classified into two groups based on whether they walked ≥ 6,000 steps/day or < 6,000 steps/day.

Each participant was given detailed information on the study protocol, and all provided their written informed consent. This study procedure was reviewed and approved by the Ethics Committee of the Graduate School of Medicine, The University of Tokyo (No.3812) and was registered in the University Hospital Medical Information Network (UMIN) Center (ID: 000008607).

2.3. *Measurements*

All participants completed a questionnaire regarding their habitual physical activities about 1 year before pregnancy and after becoming pregnant. Daily habitual physical activity was assessed using the accelerometer (*10*), which measured steps, exercise intensity, and energy expenditure. Because pregnant women avoid high-intensity exercise to prevent premature delivery, energy expenditure is proportional to the number of steps taken. Thus, steps were taken for normal life activity. The frequency of the accelerometer was 32 Hz, and its time base range was 0.06-1.94 g. As accelerometers have two axes, the detectable range is limited. No reports from previous research have indicated adverse events related to accelerometer use for pregnant women.

Casual glucose and HbA1c levels were measured to assess participants' carbohydrate metabolism. Casual glucose level was measured by the fully automatic glucose analysis apparatus GA08 (A&T Corporation, Tokyo, Japan) in the University Hospital. The blood samples for HbA1c analysis were immediately stored at 4ºC and assessed with the ADAMSA1cHA-8160 (ARKRAY, Inc., Kyoto, Japan) at FALCO Biosystems Ltd., Kyoto, Japan. The values were expressed as percentage to Hb. Oral glucose tolerance test and insulin release test were not performed in this study because the subjects were healthy pregnant women and it was difficult to ask them to fast for our study due to ethical considerations.

Dietary intake during the most recent month were assessed with a brief self-administered diet history questionnaire (BDHQ), from which we calculated the amount of daily intake for 50 foods and selected nutrients (*13,14*).

2.4. *Statistical analysis*

We consulted the previous randomized controlled trial reported by Barakat *et al*. (*15*). The influence of an exercise program performed by healthy pregnant women on maternal glucose tolerance was studied. Significant differences were found between study groups on the 50 g maternal glucose screen. Values corresponding to an exercise group (103.8 ± 20.4 mg/dL) were better than those of a control group (126.9 ± 29.5 mg/dL), $p < 0.01$. On the basis of this study, sample size was calculated with 40 pregnant women by using G*power (*16*) ($p < 0.05$ and 80% power).

The Mann-Whitney *U*-test was performed using Statistical Package Social Sciences version 19.0 (SPSS Japan Inc.). Two-tailed *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. *Participant characteristics*

Among 94 pregnant women recruited, 51 (54.3%) agreed to participate in the present study. The women who refused participation primarily stated that they did not wish to wear accelerometers. Because five women dropped out, the remaining 46 women completed the study. With respect to parity, 32 women were primiparous, and the others were in their second pregnancy (Table 1). There were no women with a pre-pregnancy BMI <18 kg/m^2 or >25 kg/m^2 or who had anemia, type 1 or type 2 diabetes or GDM during the previous pregnancy. Among them, casual glucose levels were not checked for two women in the first trimester and for one woman in the first and second trimesters, and walking data were not obtained from two women. For this reason, six women were excluded for further analyses. Thus, final data obtained from 35 pregnant women were analyzed. The average number of steps for all participants were classified into two groups; 22 women walked ≥ 6,000 steps/day, and 13 walked < 6,000 steps/day. Thus, 65.7% of the subjects walked ≥ 6,000 steps/day in the present study.

As shown in Table 1, there was no statistically significant difference in the median age, pre-pregnancy BMI, gestational week, hemoglobin level, or casual glucose level between the two groups at recruitment, around 16 gestational weeks.

3.2. *Physical activities and dietary intake*

As shown in Table 2, seven women regularly walked during the study. Women in the group walking ≥ 6,000 steps/day showed significantly higher calorie consumption (189 kcal/day) compared to those in the group that walked less (115 kcal/day). There was no statistically significant difference between the two groups with respect to energy, protein, fat, and carbohydrate intake.

3.3. *The relationship between physical activity and carbohydrate metabolism*

The relationship between the number of steps walked and casual glucose level was analyzed (Table 3). The median casual glucose level for the group walking ≥ 6,000 steps/day (83.5 mg/dL) was significantly lower than that for the group walking < 6,000 steps/day

Table 1. Characteristics at recruitment

Items	Total (n = 35) Median (IQR[a])	< 6000 steps/day (n = 13) Median (IQR[a])	≥ 6000 steps/day (n = 22) Median (IQR[a])	p value
Age (years)	36.0 (31.5, 39.0)	36.0 (26.0, 39.0)	35.5 (33.0, 37.8)	0.73[d]
pre-pregnancy BMI[b] (kg/m^2)	20.8 (19.0, 21.9)	21.5 (19.1, 22.3)	20.8 (18.7, 21.3)	0.18[d]
Primipara (n)	32	13	19	0.28[e]
Single pregnancy (n)	33	12	21	1.0[e]
Gestational weeks	16.0 (14.5, 17.0)	17.0 (14.0, 17.0)	16.0 (15.0, 17.0)	0.99[d]
Weight (kg)	52.3 (49.6, 56.6)	52.3 (49.4, 56.6)	53.3 (49.9, 56.6)	0.69[d]
Casual glucose (mg/dL)	87 .0 (81.0, 89.0)	82.0 (80.0, 87.0)	87.0 (82.5, 89.0)	0.06[d]
Hb[c] (mg/dL)	12.8 (11.5, 13.3)	12.9 (12.7, 13.4)	12.3 (11.3, 13.3)	0.33[d]

[a]IQR: interquartile range; [b]BMI: Body Mass Index; [c]Hb: hemoglobin; [d]Mann-Whitney-*U* test; [e]Fisher's exact test.

Table 2. Physical activity and dietary intakes among the investigation

Items	Total (n = 35) Median (IQR[a])	< 6000 steps/day (n = 13) Median (IQR[a])	≥ 6000 steps/day (n = 22) Median (IQR[a])	p value
Life activity				
Exercise habits (n)	5	0	5	0.13[c]
Exercise habits (days/week)	–	–	7.0 (5.9, 7.0)	–
Exercise habits (min/day)	–	–	40.0 (32.5, 40.0)	–
Step (steps/day)	6862 (5349, 8341)	5031 (4331, 5386)	8044 (6950, 8666)	< 0.001[b]
Calorie consumption (kcal/day)	161 (122, 194)	115 (98, 127)	189 (164, 207)	< 0.001[b]
Dietary intakes				
Energy (kcal/day)	1400 (1201, 1689)	1316 (1187, 1396)	1525 (1226, 1715)	0.30[b]
Protein (%E)	14.5 (13.7, 15.8)	15.2 (14.3, 15.6)	14.1 (13.3, 15.9)	0.46[b]
Fat (%E)	26.4 (24.4, 28.2)	26.3 (24.6, 29.1)	26.4 (24.3, 27.4)	0.6[b]
Carbohydrate (%E)	57.9 (56.1, 60.8)	57.6 (55.7, 60.2)	57.9 (56.4, 61.2)	0.73[b]

[a]IQR: interquartile range; [b]Mann-Whitney-U test; [c]Fisher's exact test.

Table 3. Laboratory data at the end of the investigation

Items	Total (n = 35) Median (IQR[a])	< 6000 steps/day (n = 13) Median (IQR[a])	≥ 6000 steps/day (n = 22) Median (IQR[a])	p value[d]
Gestational weeks	20.0 (19.0, 21.5)	21.0 (20.0, 22.0)	20.0 (19.0, 21.0)	0.31
Weight (kg)	54.5 (51.4, 59.0)	54.5 (51.2, 57.4)	55.4 (51.9, 58.4)	0.66
Casual glucose (mg/dL)	85.0 (80.5, 90.0)	90.0 (85.0, 96.0)	83.5 (79.3, 86.8)	0.03
HbA_{1c}[b] (%)	5.0 (4.9, 5.1)	5.0 (4.8, 5.2)	5.0 (4.9, 5.0)	0.90
Hb[c] (mg/dL)	11.6 (11.0, 11.9)	11.6 (11.0, 11.8)	11.6 (10.8, 12.0)	0.58

[a]IQR: interquartile range; [b]HbA_{1c}: hemoglobin A_{1c}. [c]Hb: hemoglobin; [d]Mann Whitney-U test.

(90.0 mg/dL; p = 0.012). After the 4-week observation period, casual glucose level in the women walking ≥ 6,000 steps/day decreased by 3.5 mg/dL, from 87.0 to 83.5 mg/dL. In contrast, that in the group walking less increased by 8.0 mg/dL, from 82.0 to 90.0 mg/dL. Thus, a large difference in the alteration of the casual glucose level (11.5 mg/dL) was observed between the two groups. On the other hand, HbA1c levels were not significantly different between the two groups.

4. Discussion

Although previous studies suggested that physical activities could be effective for management of pregnant women with GDM (*15-19*), to our knowledge, this is the first report that simple walking as a habitual daily physical activity is effective for controlling casual glucose levels in pregnant women during the second trimester. Our results suggest that daily habitual physical activity is associated with carbohydrate metabolism among pregnant women, as it is for middle-aged women (*9*).

It is known that casual glucose levels decrease easily when high-intensity exercise is performed. In a previous study, an exercise program for healthy pregnant women consisting of three sessions of aerobics and swimming per week resulted in a decrease in the casual glucose level in the intervention group (*20*). However, it can be difficult for pregnant women to begin participating in leisure sports because they usually receive a recommendation from their obstetricians to avoid high-intensity exercise in order to prevent miscarriage and premature delivery. Thus, the present study focused on daily walking as a moderate-intensity activity for healthy pregnant women. The observation that a significant difference occurred in casual glucose level based only on the number of steps walked daily is a very interesting one. We believe that walking as a habitual daily physical activity will be very attractive for pregnant women.

In a previous study, improvement in HbA1c level was directly proportional to exercise intensity, and there was no significant HbA1c improvement based on the amount of exercise (*21*). That study suggested that the casual glucose level improved in proportion to exercise intensity, not to its amount. In our research, the exercise intensity was low, as we measured walking, which may be why we did not observe an improvement in HbA1c level.

There are several limitations of this study. First, because it was conducted in one hospital with a small number of participants, our results may not be generalizable. Second, participants could not be blinded to the data from the accelerometers. The way of wearing was guided individually at the time of study, but walking steps may have been underestimated by poor wearing. Third, the influence of a meal could not be ruled out, because casual glucose levels were evaluated. However, it took at least 1 hour from check-in at the reception desk to blood collection, so this influence may have been small. Moreover, further

studies should be performed to confirm that not only blood glucose level but also insulin resistance improves by walking.

In conclusion, we found that a relationship exists between the number of steps walked per day and casual glucose level, whereas walking may prevent GDM from occurring in pregnant Japanese women.

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Case Report

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Depression in adult patients with biotin responsive basal ganglia disease

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Summary **Biotin responsive basal ganglia disease (BBGD), is a potentially treatable inherited metabolic disorder which clinically presents as sub-acute encephalopathy in children. Early diagnosis and treatment of this disorder results in good clinical recovery in childhood. However, there is no report in the literature on the long term outcome of these treated patients in adult life. We report two patients with BBGD who were metabolically stable on treatment and developed depression later in life. These cases highlight the association of depression with basal ganglia disorders and demonstrate that depression is the potential long term complication of BBGD.**

Keywords: Basal ganglia disease, depression, biotin, thiamine

1. Introduction

Biotin responsive basal ganglia disease (BBGD) is an autosomal recessive neuro-metabolic disorder, also known as thiamine metabolism dysfunction syndrome-2 (THMD2) (MIM: 607483). It was first described by Ozand *et al*. in 1998 in ten Arab patients (*1*). Later causative mutation in *SLC19A3* gene was discovered with known founder mutation in Saudi Arabia [c.1264 A>G; p.T422A] (*2*). It is a pan-ethnic disorder which has been reported in European, Indian and Japanese patients (*3,4*). It presents as encephalopathy with extrapyramidal signs in children, often following a febrile illness. Brain magnetic resonance imaging (MRI) demonstrates bilateral symmetrical involvement of the basal ganglia. Administration of biotin and thiamine early in the course of disease, reverses the clinical sign and symptoms within days in this, otherwise potentially fatal disease (*1,2*). Nevertheless, like many other inherited metabolic disorders, there is no information available on the course of BBGD in adult life. Herein, we report two siblings with BBGD who were described in the original report by Ozand *et al*. and they developed depression in adult life.

2. Case Report

2.1. *Case 1*

A 32-year-old lady, a known case of BBGD, was diagnosed at the age of 7 years when she presented with confusion, lethargy, dysarthria, dysphagia and severe spasticity of limbs. Her brain MRI demonstrated extensive bilateral T2 hyperintensity, with symmetrical involvement of the caudate nuclei and putamen. She showed remarkable response to biotin and thiamine therapy with resolution of symptoms except for persistent mild stuttering. Genetic testing later revealed homozygous mutation (c.1264A>G, p.T422A) in *SLC19A3* gene. She graduated from high school with average academic achievements. At 13 years of age in 1996, she developed mild episode of depression with no clear triggers. She continued to have recurrent episodes of mild depression till 2010 when her symptoms got worse with low mood, feeling of being isolated, poor sleep, poor appetite, fatigue and wishes of death without having any actual suicidal thoughts, intentions or plans. She lost 5 kilogram in weight

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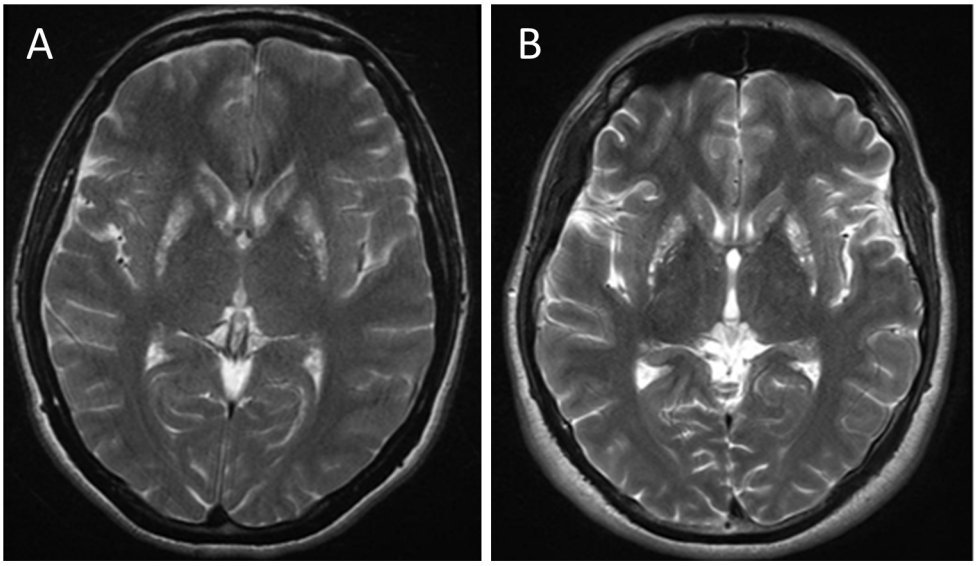

Figure 1. Magnetic resonance axial T2 weighted images showing necrosis of bilateral caudate and putamen in both patients.

during this episode, which lasted for a month. She was seen by a psychiatrist and a diagnosis of major depression was confirmed. She denied having any suicidal or homicidal ideation, manic symptoms, audiovisual hallucinations, or paranoid ideation. She was started on escitalopram but did not comply with the treatment due to the concerns about its side effects. She was later prescribed other antidepressant medications including paroxetine and paliperidone but she felt that her depression got worse with these medications and stopped treatment. Although she blames the death of her husband in 2014 in a motor vehicle accident for her depressive symptoms but is unable to explain the cause of depression starting from the younger age of 13. Her last depressive episode was two years ago. Currently, she feels better. She is sleeping and eating well. She has no symptoms suggesting generalized anxiety disorder, obsessive compulsive disorder, or post-traumatic stress disorder. She however, has social phobia. A recent brain MRI showed necrosis of bilateral caudate and putamen (Figure 1A) while magnetic resonance spectroscopy (MRS) was unremarkable.

2.2. *Case 2*

A 38-year-old gentleman, elder brother of patient 1, also a known case of BBGD, was diagnosed at the age of 12 when he presented with ptosis, dysarthria, dysphagia, and dystonic movement in the lower limbs. Based on the family history of affected younger sister with BBGD, he was started on biotin and thiamine. His brain MRI demonstrated similar changes as seen in his sister. Genetic testing confirmed the same homozygous mutation in *SLC19A3* gene. He also showed complete clinical recovery following treatment with biotin and thiamine except for mild dystonia in his left hand. At the age of 16, he developed seizures when he had stopped taking biotin. This resolved after resuming the therapy. He received bachelor's degree in the management and was employed with good performance at work. He led an active life with regular participation in sports. However, in 2007 at the age of 29, he started having low mood, isolating himself with loss of interest. He had poor interrupted sleep, fatigue, decreased concentration and loss of appetite. He lost over 20 kg in weight in a few weeks. He denied having any thoughts of death, or suicidal ideation or psychomotor retardation. These symptoms persisted for about three weeks. He was seen by the psychiatrist and treated with venlafaxine and mirtazapine with significant improvement in his symptoms. He thought that the trigger for the first episode of depression was financial difficulties due to loss of money in the stock market. However, later he continued to have mild episodes of depression. Initially he had anxiety attacks as well, with symptoms of shaking and sweating. In addition, he had obsessive compulsive symptoms that included prominent fears of contaminations, for a brief period of 6 months in 2009. It responded well to the treatment with clomipramine. There were no complaints of any phobia, generalized anxiety disorder, or post-traumatic stress disorder symptoms. He remains compliant to the maintenance therapy with venlafaxine, trazodone and clomipramine. Currently he feels very well and denies having any depressive symptoms. He is working as an accountant and continues to actively participate in the sports. His recent brain MRI (Figure 1B) also showed necrosis of caudate and putamen while MRS study was unremarkable.

3. Discussion

Depression has been reported in a wide variety of inherited errors of metabolism (IEM) including

urea cycle disorders, porphyria, homocysteinuria, phenylketonuria, GM2 gangliosidosis, Niemann-Pick C disease and Fabry disease (*5*). With our report we are expanding the list of inborn errors of metabolism that can be associated with depression.

The two siblings in this report are the eldest patients with BBGD, seen in our hospital. They had good clinical recovery following initial presentation in childhood, yet they developed depression later in life, despite adequate metabolic control. The diagnosis was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition (DSM IV) and they met the criteria for major depressive disorder. There was no recorded family history of depression.

The underlying pathophysiology of depression in IEM is still not fully understood. These patients may develop depression, secondary to the psychological and social issues related to their chronic medical illness. Nevertheless, depression may be a sequelae of the structural and functional changes in the brain secondary to their metabolic disease. Clinical and experimental data has shown basal ganglia involvement in controlling cognition and behavior in addition to movement control (*6*). Depression is well known in basal ganglia disorders like Parkinson's disease, Wilson's disease and Huntington's disease where it may precede the development of movement disorder (*7*). Basal ganglia are linked to the orbitofrontal and prefrontal cortices as well as limbic system *via* functional parallel circuits (*6*) and it has been postulated that disruption of cortico-basal ganglia connectivity due to neuronal degeneration or altered myelination may contribute to the development of depression in these patients (*5,6*). Moreover, dopamine or serotonin depletion, which affects the cortico-basal ganglia functional circuits, also contributes to the etiology of depression (*5,6*). It is interesting to note that in spite of good clinical recovery with successful treatment of BBGD, the recent brain MRI study of both patients after over 25 years of diagnosis, continues to show changes in the basal ganglia. This affirms the previous reports (*1,8*). In addition, Husain *et al*. have reported diminished volume of putamen in brain MRI of patients with major depression (*9*). Long standing basal ganglia pathology as seen in the brain MRI of both patients, therefore suggests that it may have a significant role in the development of major depression in these cases.

Despite having a chronic metabolic disorder, both patients led an active life with academic achievements prior to developing depression. The female patient first showed symptoms at the age of 13. Although she cites the death of her husband, for her symptoms, this does not explain the origin of depression since adolescence and its periodic recurrence. Similarly her brother, the second patient, tends to blame external factors like loss of income for depression. This however, does not explain why his depression started at an early age and was episodic in nature. He had required maintenance therapy with psychotropic medications for the past 8 years. Early age of onset with no triggers and recurrent pattern suggest that depression in both patients is endogenous in origin. Hence, we postulate that depression in BBGD is likely to be organic in nature secondary to basal ganglion pathology rather than a reflection of chronic disease. Further research is required to better understand the long term neuropsychological outcome of this disease in adult life.

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Commentary

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The role of biobanks in elucidating prevalent genetic diseases in Saudi Arabia

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Summary **Biobanking entails large-scale collection of human biological specimens that are linked to the donors' health and personal information, and has several applications in clinical research. Human biological specimens, such as blood, urine and tissue, have become immensely important to medical research: they offer a valuable source of genetic material that researchers can use to identify disease-associated genetic variation and to determine interactions between genes and environmental factors. Identification of genetic contributions to disease can lead to the development of new diagnostic tests and targeted treatments. Over the last decade, both common diseases and rare genetic disorders have been reported in Saudi Arabia. The need to generate extensive genetic data on these diseases has led to the establishment of several Saudi Arabian biobanks. Fortunately, these vital efforts have the support of the Saudi Government and researchers. However, the success of any biobank also requires public support and the willingness of the population to donate their biological material along with information on their medical records. Thus, the Saudi public needs to be informed of the benefits of maintaining biobanks, their participation needs to be encouraged through donation of biological material, and any public concerns regarding the confidential treatment of medical data need to be addressed. This article reviews the most common genetic diseases identified in the Saudi population, it describes biobanks and it examines how biobanks can support biomedical research in the area. Moreover, this article proposes measures that might help to increase public awareness of biobanks and the preparedness of the Saudi Arabian population to donate biological material.**

Keywords: Consanguinity, donation, willingness of donors

1. Introduction

Over the past decade, there has been remarkable interest in the study of genetic disorders in Saudi Arabia, and many researchers have highlighted the need for more intensive studies to help prevent genetic diseases in this region. In 2006, the Yörtürk Foundation declared that genetic diseases have spread among the people of Saudi Arabia (*1*). Several other studies have reported differences in the incidence of some genetic diseases in Saudi and Caucasian population (*2,3*). Moreover, inter-ethnic variations have recently been reported with regard to not only the incidence of genetic diseases but also drug pharmacokinetics (*4*). Indeed, studies comparing Middle Eastern Arabs and other populations found variations in the effective drug doses for treating several diseases. This variability in pharmacokinetics has been attributed, by the authors, to population-specific genetic, environmental, and nutritional factors (*4*). Due to these reasons, researchers in Saudi Arabia have greatly increased their efforts in studying common genetic diseases. Today, studies mainly at the King Faisal Specialist Hospital (KFSHRC) and National Guard and Health Affairs Hospital (NGHA) in Riyadh have indicated that certain genetic diseases are of substantial medical concern in Saudi Arabia (*5,6*). These studies have categorized the most prevalent genetic diseases in Saudi Arabia as inherited

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metabolic, neurological, hematological, endocrine, rheumatological, ophthalmological, and congenital malformations. Some rare genetic syndromes that are specific to the Saudi population have also been identified. The most common inherited disorders are as shown in Supplementary Table S1 (*http://ddtjournal.com/docindex.php?year=2016&kanno=4*).

The main reason for the increased frequency of genetically inherited diseases within the Saudi population has been attributed to the high rate of consanguineous marriages. Consanguineous marriages are generally defined as marriages between blood relatives; however, in genetics research, consanguineous marriages refer to marriages between first or second cousins. Consanguinity increases the risk of inherited diseases such as congenital anomalies and autosomal recessive diseases; the closer the relationship, the higher the risk. For the children of first cousins, the risk of congenital and autosomal recessive diseases is increased by 2-4% (*7*). Nonetheless, consanguineous marriages are quite common in many parts of the world, with the highest rates occurring in Africa, the Middle East, and Asia.

The average consanguinity rate in Saudi Arabia is very high, and the overall rate of consanguinity was found to be 56% in 2007, with 33.6% of marriages consisting of first-cousin marriages (*8*). A study has reported that the highest prevalence of consanguinity is in the Njad Region, which includes Riyadh, and in Makkah, Madinah, and the Eastern Province. The structure of society within these regions is largely tribal, and tradition encourages marriages between first cousins from the same tribe. However, there are significant variations in the prevalence of consanguinity between regions, as well as between rural and urban settlements (*8*). For example, consanguinity is less frequent in areas such as Jiddah, where foreigners come for Hajj and later settle and intermarry with the resident population. Surprisingly, despite recent developments in many aspects of life in Saudi Arabia, the consanguinity rates have remained unchanged over the last decade (*9*).

As long as the consanguinity problem persists in Saudi Arabian society, and genetic diseases will spread, as confirmed by Recent studies (*5,6*), biobanks must be established. Biobanks offer the possibility of studying and developing preventive measures and cures for genetic diseases using biological samples donated by affected individuals. They provide the basis for more detailed molecular and pharmacological studies of individuals with genetic diseases that are prevalent in a certain population, such as the Saudi population.

The success of biobanks depends on public support and the willingness to donate. In order for biobanks to be effective, large numbers of participants need to be recruited. Typically, biobank participants voluntarily contribute their samples for research, though in most cases public support cannot be taken for granted. If biobankers knew exactly what prospective donors were thinking, they would be able to address their concerns and increase participation by planning suitable educational programs that increase awareness of biomedical research and biobanking in particular. Over the past few years, several universities, hospitals, and biobanks around the world have surveyed people, asking what makes them want to participate in a research biobank. Among the top factors that were suggested to play a role in a donor's decision are altruism, personal and family benefits, availability of research results, and the influence of religious permission to donate biospecimens (*10*). Fortunately, there appears to be broad-based support for the biobanking process and the use of biosamples in research. A previous study on the rate of willingness to participate in biomedical research revealed a satisfactory attitude towards participation in Saudi Arabia (*11*), but more studies are needed to clarify public attitudes towards participation specifically in biobanking-related research.

This paper summarizes the most common genetic diseases in the Saudi population and it highlights the possible role that biobanks can play to support relevant biomedical research. This paper also suggests future educational and awareness programs that provide the information needed to increase public understanding and participation in biobanks.

2. Definition and function of biobanks

In order to understand the complex interactions that exist between genetic and environmental factors, large-scale studies of human populations need to be carried out. Biobanks are suitable tools for generating data needed to undertake these studies, as they contain large collections of human tissue linked to patients' medical records. They facilitate the opportunity to identify environmental risk factors or genetic variants that are associated with genetic diseases prevalent in a community. The generated data are analyzed to obtain statistically significant results correlating gene variants with disease phenotypes (*12,13*).

There are various definitions of biobanks in the literature, and one exhaustive definition is as follows: biobanks consist of large repositories of biospecimens that are linked to clinical data. They combine two major aspects of biomedical research: *i*) The capacity to collect and handle large amounts of biological material (*e.g.*, tissues, DNA, and proteins) and *ii*) The collection, storage, and integration of the clinical records of patients (*14*).

Another definition of a biobank is that it is a large set of data consisting of personal and health-related information, including medical records, family history, genetic background, and lifestyle. This set of data is linked to a set of stored tissues and cells. Biobank data differ from data stored in a patient registry or

in electronic medical records because these other repositories do not contain related biological specimens. However, such records can be complementary to biobanks, as they also provide information that can assist in human medical research, and particularly information relating to patient populations, and also general healthcare information on hereditary data and mortality.

When a biobank is established, biological samples are systematically collected from patients and/or other healthy donors. These samples are then sorted and stored, together with relevant medical information, primarily for the purpose of medical research and for research into genetic diseases.

2.1. *The history and emerging roles of biobanks*

On August 26, 1996, the research company deCODE genetics, Inc. was founded in Delaware, US, and later that year the company established its subsidiary, Íslensk erfðagreining, in Reykjavík, Iceland. The aim of deCODE genetics was to establish a gene bank by utilizing the already existing medical and genealogical records of the Icelandic population and to combine this information with genetic information obtained from tissue samples of the entire population. Their aim was to understand the genetic basis of the most common genetic diseases in the Icelandic population and to use the results to develop novel treatments. In December 1998, the Icelandic biobank was established as the world's first genomic biobank (*15*). Soon after, biobanks were established in several countries (*e.g.*, the Estonian Genome Project, the UK Biobank, Generation Scotland, and the CARTaGENE project in Quebec, Canada) (*16,17*).

Today, biobanks are established around the world as an important tool in genetic, medical, and behavioral science (*18*), and several countries now have well-developed national biobanking programs. Norway, Sweden, Estonia, Canada, and the UK are several countries with the most active biobanks, covering large populations. Furthermore, many recent efforts have been made to connect biobanks through networks that span countries and continents, such as EuroBioBank, TuBaFrost, the Public Population Project in Genomics (P3G), the Telethon Network of Genetic Biobanks, and the Biobanking and Bimolecular Resources Research Infrastructure (BBMRI) (*19*). Biobank networks facilitate the trans-national sharing of biobank resources. For example, the BBMRI project has recruited 270 organizations and has linked 20 million samples in 33 countries. Similarly, EuroBioBank created a network that offers access to relevant biological samples from biobanks across the region. The network consists of 16 biobanks in eight European countries and has an online database listing more than 440,000 human DNA, cell, and tissue samples linked to rare diseases. In addition to large-scale, national biobanks, several small-scale regional biobanks have also been established around the world. Unlike large-scale biobanks, which include population biobanks and biobanks for therapy and transplantation purposes and involve multiple investigators, ethics professionals, and lawyers, small-scale biobanks may involve a single investigator and a limited number of samples.

Apart from scale, modern biobanks also differ on the basis for the nature of their stored biological specimens, purpose, their target donors, and ownership. The biological specimens in a biobank may include one or a combination of tissues, macromolecules, bodily fluids, or organs. Sample types currently being stored in biobanks are blood, the buffy coat, plasma, serum, frozen tissue, buccal cells, formalin-fixed or paraffin-embedded tissues, cultured cells, donor-derived cell lines, proteins, RNA, and DNA.

The utility of a biobank can be for diagnostic purposes (such as pathology, forensics, or transplantation), for therapeutic treatment (*e.g.*, blood banks, bone marrow, cord blood, and stem cells), or for pure research. The research can be focused on a specific population or on a specific disease, such as cancer, diabetes, heart disease, or a genetically inherited disease (*20*). Thus, the target population of a biobank can be either healthy people, such as all newborns, adults, or pregnant women, or patients with a specific disease. Disease-oriented biobanks are normally linked to a hospital, where specialized staff members collect samples that represent different diseases to help with the identification of biomarkers linked to a disease (*21*). Population-based biobanks need not be linked to a particular hospital because they collect their samples from a variety of donors from the general population (*e.g.*, when conducting research on biomarkers for disease susceptibility) (*22*), and the participants may be of different ages, ethnicities, and exposed to different environments.

The ownership of a biobank can be private, public, or a partnership spanning multiple sectors. Biobanks can be run by the government, non-profit organizations, or commercial companies or by hospitals or universities (*23*). Different biobanks have different approaches to coding, privacy, and the extent to which data linkage is possible.

3. Saudi Arabian biobanks and their role in biomedical research

The high incidence of rare genetic diseases in Saudi Arabia, partly due to the custom of consanguineous marriage, has increased the need to establish Saudi biobanks that fully cater to and are closely tied to local needs. Currently, there are several biobanks in Riyadh, Saudi Arabia. These include: the Biological Repository Center at the King Faisal Specialist Hospital

(KFSHRC); the Saudi Biobank at the King Abdullah International Medical Research Center of the National Guard & Health Affairs (NGHA); and the Eye Bank at the King Khaled Eye Specialist Hospital (KKESH). The procedures Saudi biobanks follow to receive, process, store, discard, and release samples have been adapted from different established international biobanks. As an example, the NGHA biobank follows the procedures of the UK Biobank. All Saudi biobanks have departmental policies and procedures (DPP) that apply to all biobank members. DPP describe the policies and procedures for efficient and effective management of the biobank and cover in detail all of the steps involved in processing samples. Moreover, all Saudi biobanks, like international biobanks, are obligated to obtain informed consent from all participants prior to participation. Withdrawal of consent at any time is a right granted to participants (*27*).

The Biological Repository Center was established in 2004, and its primary function is the preservation and storage of archived frozen tumor and normal tissue samples. These samples will be used for DNA and RNA extraction and for subsequent analysis, including mutational analyses and differential expression analyses (*24*). The NGHA Saudi Biobank, which was established in 2007, aims to conduct an extensive study on the influence of genes, the environment, and lifestyle in common diseases (*25*). The Biobank includes four sub-biobanking facilities: the DNA Bank; the Community Biobank; the Disease Biobank; and the Tissue Biobank. The Community Biobank stores biological material from healthy individuals, whereas the Disease Biobank stores biological material from individuals with various genetic and chronic diseases. The Tissue Biobank stores breast, colon, kidney, liver, lymph node, and thyroid tissues. The Saudi Bio-bank Project plans to recruit 200,000 volunteer patients from NGHA hospitals and clinics in order to study the most common diseases in Saudi Arabia (such as diabetes, cancer, coronary artery disease, hepatitis, obesity, bronchial asthma, chronic renal impairment and failure, stroke, and inherited genetic diseases (*25*). The Eye Bank was established in 1984 for eye donation, and it primarily provides tissues for KKESH surgeons, who perform more than 400 corneal transplantations annually (*26*).

Until recently, all medical treatments in Saudi Arabia were developed through the study of non-Saudi populations. Moreover, there has been a lack of large-scale research based on populations in the region. Since many Saudi biobanks have been established or in the process of being established, scientists are obtaining access to Saudi biological samples along with health information from Saudi patients and healthy volunteers. Records of the bio-bank participants' lifestyles, gathered via questionnaires, interviews, and physical examinations, constitute the initial Saudi bio-bank database. This knowledge will allow researchers to better understand diseases that affect Saudi populations and will lead to tailored healthcare and personalized medicine.

4. Social and ethical issues associated with the willingness of donors in Saudi Arabia and the success of biobanks

The need for significant statistical results correlating gene variants and disease phenotypes necessitates the presence of large numbers of volunteers and a respective large-scale collection of biospecimens (*12,13*). Many successful biobanks pool large numbers of samples and related data by recruiting large numbers of participants who voluntarily contribute samples for research. deCODE genetics has banked the genetic samples of 100,000 Icelandic volunteers that are linked to both the Icelandic Health Sector Database and genealogical records (*15*). Similarly, the Estonian Genome Project is establishing a national genetic/medical database of one million volunteers (*16*). The National Institute of Health (NIH/USA) has facilitated biobanking by developing a centralized data repository, the database of Genotypes and Phenotypes (dbGaP). In addition, the NIH has outlined several data-sharing procedures that will allow researchers to share data obtained from NIH-supported genome-wide association studies (GWAS) (*28*).

As long as the success of biobanks depends primarily on public support and willingness, biobanks need to adopt mechanisms to maximize public interest, to maintain trust between the donors and the biobank, and to encourage people to donate samples and their medical information for research purposes (*29*). Individuals who contribute their tissues and personal information also need to feel secure in their involvement with the biobank. Generally, people have a positive opinion towards genetic research (*30-32*), but they may still harbor a negative attitude towards their own participation and sample donation (*33,34*).

There are considerable social and ethical issues associated with the functions of biobanks in Saudi Arabia. There, religion, tradition, and public awareness can greatly affect people's attitudes towards participation. These issues need to be addressed for the Saudi Arabian population and the preparedness of the Saudi people to participate in biobanks needs to be assessed so that Saudi biobanks can be established.

4.1. *Willingness of donors in Saudi Arabia to participate in comparison to Western donors*

In Western countries, several studies have addressed the public's level of willingness and the reasons that encourage volunteers to participate in biobanks or in other forms of genetic research (*32,35,36*). Western populations have more access to higher education and

are generally considered to be more familiar with the link between healthcare and research. The public are willing to participate when they understand and believe in the benefits of genetic research, and they are more likely to donate biological material to biobanks if they are assured that their information will be treated with confidentiality and they are aware that there will be no unwarranted access to their samples and information (*37*).

A previous study in the US by Kelly *et al*. (*35*) reported that altruism is one of the primary reasons for public participation in biobanks, as the majority of participants wanted to make a positive contribution to research after facing an illness themselves. Another motivation was the desire to contribute to scientific and medical knowledge and to assist researchers in identifying genes that might aid in the treatment of incurable disease. The authors indicated that ease of participation, where no additional blood samples or time was required, was also an important factor for encouraging donation of biological material to biobanks. Studies among the general population in Finland showed that the willingness to participate was associated with a belief in the benefits of genetic research (*38-40*). A similar study in Singapore reported different reasons for donation and willingness (*41*); the authors found that the most common reasons for the willingness to donate blood were: for medical advancement (81.9%), to benefit future generations (81.1%), and to create employment in life-science research (40.4%). They also reported that the reasons for not donating blood included the fear of pain, needles, injections, and blood (38.1%); no self-benefit (24.8%); the fear of finding out that they have a disease (22.3%); and the fear of discrimination by employers and insurance companies (18.7%).

Few efforts have been made to assess the concerns and willingness of Saudi individuals to participate in the country's current biobanks, and little is known about the reasons that might influence their willingness to do so (*11*). Information on public attitudes to biobank participation in the literature cannot be readily applied to the Saudi population because of the variability in education, culture, traditions, and basic assumptions. Most Saudis, excluding the educated sector, are not fully cognizant of the benefits of participation in genetic research. The general public is unfamiliar with the concept of genes, DNA, or genetic research; therefore, these issues need to be addressed before encouraging widespread public participation in the nation's biobanks. Interestingly, however, the results of two recent surveys conducted at Abdulaziz Medical City in Riyadh, Saudi Arabia indicated that the public is highly willing to contribute to genetic research (87%) and most respondents appear to have positive attitudes towards donation (*11,42*). This high level of willingness is consistent with that found in studies conducted previously in highly developed countries, such as Sweden and the US (*31,32*).

The similarity between the Swedish, American, and Saudi Arabian populations with regard to the relatively high level of willingness to donate, despite social, cultural, and educational differences, can be explained by findings presented by David Wendler, who summarized the data obtained from 30 studies and more than 33,000 individuals. His studies yielded consistent findings, despite the different nationalities of the participants, and he found that most participants favor donation and biobank participation (*43*). In addition, a comparison of the factors associated with willingness among groups that are aware of genes versus those that are not yielded very similar results for both groups (*43*). In order to encourage Saudi willingness to participate in biobanks, social, religious, and ethical factors need to be address and public awareness of biobanks needs to be increased. The tribal structure that deeply influences people's social behaviour, beliefs, and ethics is specifically influential and detrimental to the development of biobanking.

4.2. *Culture and religion*

The cultural setting in Saudi Arabia is deeply religious, conservative, traditional, and family-oriented. Islam is the state religion and the source of the legal system. Islam is turned to for guidance in all aspects of life, including research and medical issues. Saudi people respect their religious leaders and are guided by religious teachings. Accordingly, Saudi biobanks should be designed in a manner to respect not only international guidelines but also Islamic/Sharia values. Muslims' religious and cultural attitudes have to be taken into account when considering issues such as informed consent, privacy, and concerns about justice (*42,44*). Islamic sources will be consulted to ensure that the biobank is compliant with and observant of Islamic Sharia law. These sources include the Quran, Sunnah, and fatwas, which are legal opinions concerning a specific matter in Islamic law handed down by an Islamic religious leader. Both researchers and Islamic medical organizations use these institutional fatwas when they encourage participation. However, Saudi biobanks need more specialized fatwas to clarify Islamic judicial views on many issues, including informed consent, privacy, and confidentiality. Fatwas that might hinder harmonization with international biobank standards need to be avoided (*45*). A recent study in Jordan, a neighboring Muslim country, found that religious permission had a strong positive influence on the respondent decision to participate (*46*). In Saudi Arabia, very little research has been done on the effect of religion on the willingness of people to allow use of their private information and biological samples for research (*11,42*). As religious institutions have a social

authority in the Saudi society, they can effectively collaborate with biobank personnel to encourage the Saudi people to accept the types of ethical guidelines that are widely followed by healthcare professionals worldwide.

4.3. *Ethics*

An essential component of establishing a Saudi biobank is to set an ethical framework that gains public trust and consent and that facilitates donor recruitment with an ethics review board. The ethical framework should address the concerns of the donors and the principles, policy, research rules, and guidelines of biobanks.

Many countries have developed national and international guidelines for biobanks, such as the UK Biobanks Governance national guidelines (*47*), the Guidelines for Human Biobanks and Genetic Research Databases from the Organization for Economic Co-operation and Development OECD (*48*), and the International Declaration on Human Genetic Data by the United Nations Educational, Scientific, and Cultural Organization (UNESCO) (*49*).

Despite the fact that there has been a lack of published guidelines on genetic or general research ethics with regard to specimen donation in most countries of the Eastern Mediterranean region, Saudi Arabia is fortunate in already having special national guidelines concerning research ethics and efforts are underway to define all research ethics. Different documents regulating research ethics have been drafted, including general guidelines for biomedical research and official legal documents issued by the Council of Ministers. The Saudi Food and Drug Authority (SFDA) guidelines contain 15 of the protections mentioned in international guidelines on research ethics, such as the ICH-GCP, the CIOMS guidelines, and the Declaration of Helsinki (*45*). However, national regulations on research ethics and guidelines in Saudi Arabia have some deficiencies in comparison to their international counterparts (*45*). In most Saudi biobanks, *e.g*. NGHA biobank, the Institutional Review Board (IRB) is an independent body consisting of medical, scientific, and non-scientific members whose responsibility in part is to oversee ethical issues such as protection of the rights, safety, and well-being of human subjects involved in research and regulations on the use of donated samples. As all biobanks exist within institutions, whether they be academic or medical, the biobank IRB work together with the institution' ethical review board to approve all biobanking and to make decisions about biobank access for research.

Gaining donors' trust and confidence is essential for Saudi biobanks to function and it requires, in addition to well-established and clear ethical policies and rules, close collaboration between clinicians, researchers, and information technologists. Fortunately, substantial efforts are being made by the Saudi Government and researchers to address this issue and provide biobanks with expert medical personnel or clinicians to collect biospecimens and to properly manage the samples before storage. In addition to the proper handling and transportation of samples, a pathology review, and the use of qualified laboratories for subsequent molecular biology analyses, such as DNA extraction or the collection of any blood constituent, Saudi donors need to be informed of most, if not all, of the safety procedures that will be followed regarding their donated samples.

In addition, the secure storage of patient medical data and information should be ensured by the biobank. Donors should be informed of the absolute confidentiality of their medical records. The social characteristics and tribal structure of Saudi Arabia still strongly influence people's attitudes regarding their privacy and necessitate strict confidentiality regarding their personal issues. Accordingly, Saudi biobanks should adopt different mechanisms to guarantee the confidentiality of personal information. The stored samples and data should be coded and/or anonymized and kept under strict control and protected by a good security system. The data collected from medical records, medical workers, interviews, or other sources should be formatted for secure storage in a modern coded digital format. All of the digital formats must also allow easy and rapid access to code and decode the information used on specimen labels. Modern types of optically accessible barcoding systems are becoming widespread in all types of massive data storage, and this is especially true for biobanks (*50*). Finally, infrastructure would ideally be in place before any data are collected, since it will become increasingly important when the size and scope of the biobank increases.

4.4. *Education on the usefulness of biobanks*

Educational programs that clarify the benefits of participation in genetic research need to be instituted and individual fears and concerns about sample donation need to be addressed in order to establish effective and functional biobanks in Saudi Arabia. As the decision to participate is often made in concert with one's extended family, educational programs should include all family members. A general public education program can be implemented in schools by incorporating basic information into the early educational curriculum. Providing the public with genetic counseling services is also crucial, as is increasing public awareness with the help of mass media.

5. Conclusion and prospects for the future

Biobanking has the potential to be of significant benefit

to genetic research in Saudi Arabia by providing researchers in the country with the opportunity to study and draw conclusions about the role(s) of genes in the development of diseases. The true value of a biobank in Saudi Arabia is likely to be realized only in a climate of cooperation and sharing of resources and experience, both domestically and internationally.

The time has come, in Saudi Arabia, to encourage public discussion to promote the future establishment of a functional biobanking system with clearly defined pathways for obtaining consent from participants, collecting, transporting, and storing samples, and secure data analysis and retrieval. Success in this endeavor will ultimately facilitate a number of valuable research projects. Saudi Arabia can make a unique contribution to the field of genetic disease research, given the prevalence of several inherited conditions in the area and the familial structure. Concerted and well-coordinated efforts are required to increase public understanding and to address any potential concerns regarding genetic research. The public must be educated and encouraged to participate in genetic research, and the public needs to be involved in the development of a genetic research policy. Saudi researchers and the Saudi public are known to have a strong sense of social responsibility and a desire to comply with religious teachings. Thus, all biobanking activities need to be redesigned and reframed to fully comply with Sharia guidance. Accordingly, approvals by or fatwas from prominent clerics in support of biobanking will be crucial to biobanking efforts.

The establishment of highly functional biobanks in Saudi Arabia in collaboration with other biobanks worldwide, and particularly in neighboring Arab countries, will encourage future collaboration and greater discussion and harmonization of guidelines. A broader biobank program in Saudi Arabia will greatly enhance and support efforts to research diseases in surrounding Arab countries.

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