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Synthesis of Benzoxazole Amides as Novel Antifungal Agents against *Malassezia Furfur*

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Malassezia is a pathogenic fungus that causes skin diseases, such as tinea versicolor, atopic dermatitis and fatal sepsis. We report the synthesis of a series of benzoxazole amides and evaluation of their antifungal activity against *Malassezia furfur*. Twelve benzoxazole amides were prepared through the cyclization of the substituted 2-hydroxy aniline with *N*-(bis-methylsulfanylmethylene) amides. Among the prepared compounds, the compounds **4a**, **8b**, **8c** and **8d** showed *in vitro* antifungal activity.

Key Words: Benzoxazole amides, Antifungal, *Malassezia furfur*

Introduction

Malassezia furfur is a pathogenic fungus that causes skin diseases, such as tinea versicolor and fatal sepsis. *Malassezia* species have been recognized as members of the microbiological flora of animal and human skin.[1] And they are also considered as pathogenesis of atopic dermatitis,[2,3] seborrheic dermatitis,[4] folliculitis and otitis externa.[5-7] As most of *Malassezia* species show lipid-dependency, lipolytic enzymes including lipase and phospholipase are necessary for them to obtain useful lipids from the environment. Consequently, these enzymes are thought to play an important role in the growth and pathogenicity of *Malassezia*. *Malassezia* activates macrophages to produce inflammatory cytokines or chemokines.[8]

Malassezia furfur pathogenecity is associated with the production of bioactive indoles. Malassezin (Fig. 1) is a tryptophan metabolite isolated from the *M. furfur*. Melassezin induces apoptosis in cultured human melanocytes through activation of the aryl hydrocarbon receptor (AhR) and it was suggested that it could be easily converted to the potent AhR ligand indolo[3, 2-b] carbazole (ICZ) (Fig. 1). It has been reported that a pathogenic model of seborrheic dermatitis is associated with ICZ- and malassezin-mediated AhR activation.[9]

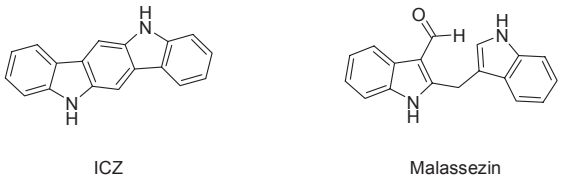


Figure 1. Chemical structures of ICZ and malassezin.[9]

Malassezia-associated skin disease is one of the major classes of superficial cutaneous mycotic infections caused by several different *Malassezia* species. *Malassezia*-associated skin disease is usually subjected to topical antifungal treatment. Therefore, an antifungal agent of topical use should be good to treat against such *Malassezia* species.[10]

It has been reported that various antifungal agents including fluconazole, itraconazole, ketoconazole, voriconazole and terbinafine are active *in vitro* against *Malassezia* species. However, the *in vitro*/*in vivo* relationship of the anti-*Malassezia* activity of antifungal drugs or the pathogenetic role of each *Malassezia* species in the development of *Malassezia*-associated skin diseases remains to be answered.[11]

Benzoxazole is structurally related to biologically important bases, and constitutes a class of heterocyclic compounds exhibiting substantial therapeutic activities including antibiotic, antimicrobial, antiviral, topoisomerase I and II inhibitors, and antitumor activities.[12-17] Various 2-(substituted phenyl or benzyl) benzoxazole derivatives have been reported to have antifungal activity (Fig. 2).[12,13] Also, benzoxazole amide scaffold is found in a number of marketed drug formulations, and reported as the treating dyslipidemia or arteriosclerotic diseases.[18]

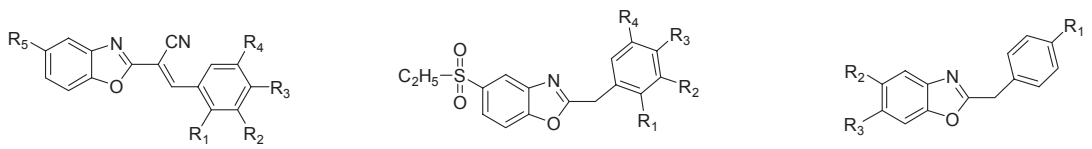


Figure 2. Reported benzoxazole derivatives as antifungal agents.[13-15]

[a]These authors equally contributed to this work.

Based on the chemical structure of malassezin and reported benzoxazole derivatives, we designed and synthesized benzoxazole amides and evaluated their antifungal activity against *Malassezia furfur*. The goal of this research is to elucidate the new effective antifungal agents with simple structure and easy synthetic route. Herein, we describe our preliminary lead optimization efforts culminating in the identification of a novel series of benzoxazole amide with antifungal activity.

Results and Discussion

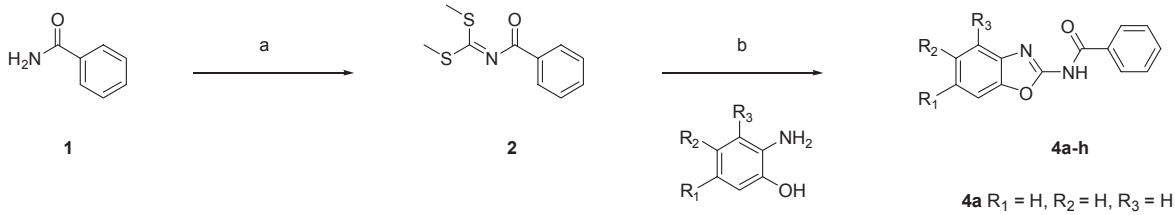
Chemistry. Twelve benzoxazole amide derivatives (**4a-h** and **8a-d**) were prepared from the cyclization of substituted 2-hydroxyaniline with *N*-(bis-methylsulfanylmethylene) amides by simple modification of reported method as illustrated in Schemes 1 and 2.[19,20] The corresponding *N*-(bis-methylsulfanylmethylene) amide derivatives (**2** and **6a-d**) were obtained from the reaction of substituted benzamides with carbon disulfide, iodomethane and sodium hydride, through *in situ* methylation of congener monoesters. This procedure was simple and mild reaction with 25 - 90% yields. Compare to this, the general method for the synthesis of benzoxazole amide derivatives requires a substituted 2-aminobenzoxazol which reacts with the substituted benzoic acid or substituted benzoyl chloride.[21] A substituted 2-aminobenzoxazol is usually prepared from substituted 2-amino phenol and BrCN. And BrCN is sensitive to moisture, volatile and toxic when absorbed through the skin.

Antifungal activity. All the synthesized benzoxazole amide derivatives were evaluated *in vitro* for antifungal activity against *Malassezia furfur* with inhibition zones and MIC values.

At first examination, we performed antifungal studies using the disc diffusion method. Compounds **4a**, **8b**, **8c** and **8d** showed antifungal activity with clear zone of 15, 8, 10 and 12 mm, respectively. The marketed antifungal agent ketoconazole was used as positive control which showed clear zone of 42 mm.

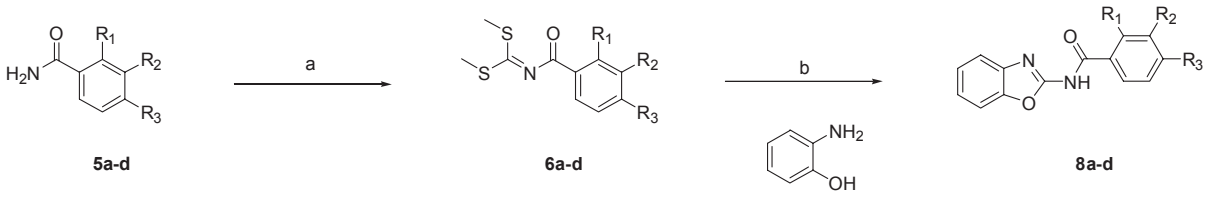
MIC values of synthesized compounds were determined by broth microdilution. As shown in Tables 1 and 2, inhibitory activity of synthesized compounds was changed by the position of substitution. The compound without substitution for both aromatic rings (**4a**) showed highest inhibitory activity with MIC value of 350 μg/mL. Compounds derived from substituted benzoxazole rings (**4b-4h**) showed no antifungal activity. However, the congeners with nitro, methyl or methoxy substitution on para-position of benzene ring (**8b-8d**) showed mild antifungal activity with MIC value of > 1,000 μg/mL. However *p*-*N*-(bis-methylsulfanylmethylene) amino group substituted compound (**8a**) showed no inhibitory activity.

Cytotoxicity. To evaluate cytotoxicity of the synthesized compounds, MTT assay was performed. The cytotoxicity of prepared compounds was measured using HepG2 cell line. As shown in Table 3, three compounds (**4a**, **8b**, and **8d**) among the compounds have antifungal activity (**4a**, **8b**, **8c**, and **8d**) showed low toxicity at 10 ug/mL. The viability of **4a**, **8b**, and **8d** was 42, 43, and 34%, respectively. Compared to the viability of keto-



4a R_1 = H, R_2 = H, R_3 = H
4b R_1 = H, R_2 = Cl, R_3 = H
4c R_1 = H, R_2 = NO_2, R_3 = H
4d R_1 = H, R_2 = OCH_3, R_3 = H
4e R_1 = H, R_2 = H, R_3 = CH_3
4f R_1 = CH_3, R_2 = H, R_3 = H
4g R_1 = H, R_2 = COOH, R_3 = H
4h R_1 = CF_3, R_2 = H, R_3 = H

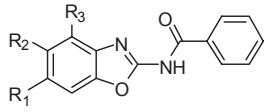
Scheme 1. Reagents and conditions: (a) CS_2, MeI, NaH, DMF, rt, 5h; (b) DMF, Reflux, 5h



8a R_1 = H, R_2 = $C_3H_6NS_2$, R_3 = H
8b R_1 = Cl, R_2 = H, R_3 = NO_2
8c R_1 = H, R_2 = H, R_3 = CH_3
8d R_1 = H, R_2 = H, R_3 = OCH_3

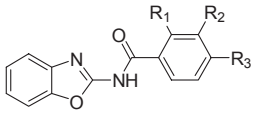
Scheme 2. Reagents and conditions: (a) CS_2, MeI, NaH, DMF, rt, 5h; (b) DMF, Reflux, 5h

Table 1. The minimal inhibitory concentrations (MIC) of **4a-h** against *M. furfur*



Compounds	R_1	R_2	R_3	Clear zone (mm)	MIC (μg/mL)
4a	H	H	H	15	350
4b	H	Cl	H	-	-
4c	H	NO_2	H	-	-
4d	H	OCH_3	H	-	-
4e	H	H	CH_3	-	-
4f	CH_3	H	H	-	-
4g	H	COOH	H	-	-
4h	H	CF_3	H	-	-
ketoconazole				42	16

Table 2. The minimal inhibitory concentrations (MIC) **8a-d** against *M. furfur*



Compounds	R_1	R_2	R_3	Clear zone (mm)	MIC (μg/mL)
8a	H	$C_3H_6NS_2$	H	-	-
8b	Cl	H	NO_2	8	>1000
8c	H	H	CH_3	10	>1000
8d	H	H	OCH_3	12	>1000

Table 3. Cytotoxicity of prepared compounds at 10 ug/mL using HepG2 cell line

Compounds	Viability %
4a	42.84
4b	10.54
4c	10.56
4d	4.81
4e	33.81
4f	45.73
4g	50.36
4h	5.66
8a	28.11
8b	41.95
8c	6.78
8d	34.03
ketoconazole	72.13

conazole (72%), three compounds were fairly low toxic on Hepatitis cell line. However, compound **8c** showed higher toxicity than others with viability 7% at 10 ug/mL. There was no relationship of cytotoxicity and antifungal activity on the prepared benzoxazole amides.

Conclusion

It has been a major expansion in the development of antifungal drugs, but there are still weaknesses in the range and scope of current antifungal chemotherapy. New developments have included modification of existing drug molecules to minimize toxicity and improve activity For this purpose, we synthesized a series of benzoxazole amide derivatives and examined antifungal activity against *M. furfur*. Their activity was lower than that of ketoconazole, but this result could be used for the design of new antifungal agents with simple in structure and easy to prepare.

Experimental

Materials and methods. Melting points were measured on an electro thermal digital melting point (Buchi, Germany) without calibration. ^{1}H NMR spectra were recorded on Varian NMR AS and Varian Unity Inova 400 MHz NMR spectrometers. Chemical shifts were reported in parts per million (δ) units relative to the solvent peak. The ^{1}H NMR data were reported as peak multiplicities: s for singlet; d for doublet; t for triplet; and m for multiplet. Coupling constants were recorded in hertz. MS spectra were measured using Jeol JMS 700 high resolution mass spectrometer from the Korea Basic Science Institute (Daegu). Reagents were of commercial grade and were purchased from Sigma-Aldrich Co., Merck, Ducksan Pure Chemical Co.

General procedure for *N*-(bis-methylsulfanyl methylene) substituted benzamides. A solution of substituted benzamide (16.51 mmol), carbon disulfide (66.04 mmol), iodomethane (52.83 mmol), and 60.0% sodium hydride dispersion in mineral oil (33.02 mmol) in *N*,*N*-dimethylformamide was stirred at room temperature for 5 h. The reaction mixture was dissolved in ice water, and extracted with EtOAc. The organic phase was combined, dried over anhydrous $MgSO_4$, concentrated *in vacuo*, and purified by chromatography on a silica gel using *n*-hexane/EtOAc = 7:1 to afford the products.

***N*-(Bis-methylsulfanyl methylene) benzamide (2):** Yield 41%; yellow powder; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.10 - 8.12 (2H, m), 7.62 (1H, t, J = 7.4 Hz), 7.50 - 7.54 (2H, m), 2.63 (6H, s).

***N*-(Bis-methylsulfanyl methylene)-3-bis-methylsulfanyl methylene benzamide (6a):** Yield 4%; yellow powder; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 7.81 - 7.85 (1H, m), 7.56 - 7.57 (1H, m), 7.47 (1H, t, J = 7.8 Hz), 2.53 (6H, s), 7.07 - 7.10 (1H, m), 2.63 (6H, s).

***N*-(Bis-methylsulfanyl methylene)-2-chloro-4-nitrobenzamide (6b):** Yield 18%; yellow powder; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.34 (1H, d, J = 2.4 Hz), 8.28 (1H, dd, J = 8.6, 4.4 Hz), 8.20 (1H, d, J = 8.6 Hz), 2.66 (6H, s).

***N*-(Bis-methylsulfanyl methylene)-4-methylbenzamide (6c):** Yield 18%; white powder; ^{1}H NMR (Acetone-d_6, 400 MHz) δ

7.99 (2H, d, J = 7.2 Hz), 7.33 (2H, d, J = 7.2 Hz), 2.62 (6H, s), 2.41 (3H, s).

***N*-(Bis-methylsulfanyl methylene)-4-methoxybenzamide (6d):** Yield 29%; white powder; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.06 (2H, d, J = 6.8 Hz), 7.03 (2H, d, J = 9.2 Hz), 3.89 (3H, s), 2.61 (6H, s).

General procedure for the synthesis of compounds 4a-h and 8a-d. To a stirred solution of **2** (100 mg, 0.44 mmol) dissolved in DMF was added **3a** (48 mg, 0.44 mmol) at reflux for 5h. After cooling, the solvent was removed *in vacuo* and the residue was washed with Et_2O to provide **4a**.

***N*-(Benzo[*d*]oxazol-2-yl)benzamide (4a):**[22] Yield 76%; pale yellow powder; mp 190.3 - 192.1 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.20 (2H, d, J = 7.2 Hz), 7.51 - 7.63 (5H, m), 7.30 - 7.37 (2H, m); FABHRMS *m/z* 239.0817 (MH^+, $C_{14}H_{10}N_2O_2$ requires 239.0821).

***N*-(5-Chlorobenzo[*d*]oxazol-2-yl)benzamide (4b):**[22] Yield 31%; pale brown powder; mp 214.6 - 218.3 °C; ^{1}H NMR (Acetone- d_6, 400 MHz) δ 8.30 (1H, d, J = 2.4 Hz), 8.02-8.03 (2H, m), 7.68 (1H, d, J = 8.4 Hz), 7.66 (1H, m), 7.55 (2H, m), 7.32 (1H, dd, J = 8.4, 2.4 Hz),; FABHRMS *m/z* 273.0431 (MH^+, $C_{14}H_{10}ClN_2O_2$ requires 273.0431).

***N*-(5-Nitrobenzo[*d*]oxazol-2-yl)benzamide (4c):** Yield 29%; pale yellow powder; mp 204.9 - 207.1 °C; ^{1}H NMR (DMSO, 400 MHz) δ 8.46 (1H, d, J = 2.0 Hz), 8.24 (1H, dd, J = 9.2, 2.0 Hz), 8.04 - 8.06 (2H, m), 7.92 (1H, d, J = 9.2 Hz), 7.65 - 7.69 (1H, m), 7.55 - 7,59 (2H, m); FABHRMS *m/z* 284.0675 (MH^+, $C_{14}H_{10}N_3O_4$ requires 284.0671).

***N*-(5-Methoxybenzo[*d*]oxazol-2-yl)benzamide (4d):** Yield 45%; pale brown powder; mp 162.4 - 166.2 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.18 - 8.20 (2H, m), 7.60 (1H, m), 7.50 - 7.54 (2H, m), 7.44 (1H, d, J = 8.8 Hz), 7.14 (1H, d, J = 2.4 Hz), 6.88 (1H, dd, J = 8.8, 2.4 Hz), 3.85 (3H, s); FABHRMS *m/z* 269.0929 (MH^+, $C_{15}H_{13}N_2O_3$ requires 269.0926).

***N*-(6-Methylbenzo[*d*]oxazol-2-yl)benzamide (4e):** Yield 80%; white powder; mp 204.2 - 206.0 °C; ^{1}H NMR (DMSO, 400 MHz) δ 8.04 - 8.06 (2H, m), 7.59 (1H, m), 7.49 - 7.52 (2H, m), 7.40 - 7.42 (2H, m), 7.10 (1H, brd, J = 8.0 Hz), 2.41 (3H, s); FABHRMS *m/z*: 253.0976 (MH^+, $C_{15}H_{13}N_2O_2$ requires 253.0977).

***N*-(4-Methylbenzo[*d*]oxazol-2-yl)benzamide (4f):** Yield 28%; yellow powder; mp 141.6 - 142.2 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.93 (2H, m), 7.55 (1H, m), 7.43 - 7.47 (2H, m), 7.24 (1H, brd, J = 7.6 Hz), 7.10 (1H, t, J = 7.6 Hz), 7.04 (1H, brd, J = 7.6 Hz), 2.47 (3H, s); FABHRMS *m/z* 253.0983 (MH^+, $C_{15}H_{13}N_2O_2$ requires 253.0977).

2-(Benzamido)benzo[*d*]oxazole-5-carboxylic acid (4g): Yield 81%; white powder; mp > 280 °C; ^{1}H NMR (DMSO, 400 MHz) δ 8.11 (1H, d, J = 1.6 Hz), 8.05 - 8.07 (2H, m), 7.94 (1H, dd, J = 8.4 and 1.6 Hz), 7.74 (1H, d, J = 8.4 Hz), 7.65 (1H, m), 7.54 - 7.57 (2H, m); FABHRMS *m/z*: 283.0716 (MH^+, $C_{15}H_{11}N_2O_4$ requires 284.0719).

***N*-(5-(Trifluoromethyl)benzo[*d*]oxazol-2-yl)benzamide (4h):** Yield 54%; pale brown powder; mp 182.6 - 183.9 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.91 - 7.93 (2H, m), 7.77 (1H, brs), 7.59 (1H, d, J = 8.4 Hz), 7.50 - 7.57 (2H, m), 7.42 - 7.46 (2H, m); FABHRMS *m/z* 307.0691 (MH^+, $C_{15}H_{10}F_3N_2O_2$ requires 307.0694).

Dimethyl 3-(Benzo[*d*]oxazol-2-ylcarbamoyl)phenylcarbonimidodithioate (8a): Yield 89%; brown powder; mp 185 - 186 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 7.92 (1H, brs), 7.67 (1H, brs), 7.58 - 7.60 (1H, m), 7.55 - 7.57 (1H, m), 7.48 (1H, t, J = 7.8 Hz), 7.31-7.39 (2H, m), 7.08 (1H, d, J = 7.6 Hz), 2.54 (6H, s); FABHRMS *m/z* 358.0685 (MH^+, $C_{17}H_{15}N_3O_2S_2$ requires 358.0684).

***N*-(Benzo[*d*]oxazol-2-yl)-2-chloro-4-nitrobenzamide (8b):** Yield 76%; brown powder; mp 204.1 - 205.5 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.37 (1H, d, J = 2.0 Hz), 8.33 (1H, dd, J = 2.2, 8.6 Hz), 8.10 (1H, d, J = 8.4 Hz), 7.58 - 7.60 (2H, m), 7.34 - 7.41 (2H, m); FABHRMS *m/z* 318.0283 (MH^+, $C_{14}H_8ClN_3O_4$ requires 318.0282).

***N*-(Benzo[*d*]oxazol-2-yl)-4-methylbenzamide (8c):**[18] Yield 66%, pale brown powder; mp 199 - 199.9 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.09 (2H, d, J = 8.0 Hz), 7.54 - 7.59 (2H, m), 7.30 - 7.38 (4H, m), 2.43 (3H,s); FABHRMS *m/z* 253.1057 (MH^+, $C_{15}H_{12}N_2O_2$ requires 235.0633).

***N*-(Benzo[*d*]oxazol-2-yl)-4-methoxybenzamide (8d):**[18] Yield 84%; brown powder; mp 173 - 173.5 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.18 (2H, d, J = 6.8 Hz), 7.53 - 7.58 (2H, m), 7.29 - 7.37(2H, m), 7.07 (2H, d, J = 8.4 Hz), 3.90 (3H, s); FABHRMS *m/z*: 269.0928 (MH^+, $C_{15}H_{12}N_2O_3$ requires 269. 0926).

Antifungal activity assay. The standard reference yeast, *Malasseaia furfur* CBS1878 was purchased from CBS Fungal Biodiversity Centre, Netherland. The medium used was Sabouraud dextrose agar (SDA; Difco, USA). The broth culture of *Malasseaia furfur* was harvested and suspended in PBS buffer solution, and agitated a vortex mixer for one hour. 100μL of the test samples were spread onto the Sabouraud agar plates. Filter paper discs (Whatman No. I) impregnated with 200 μg/10 μL concentration of chemicals were applied to the agar plates on which *Malasseaia furfur* had been overlaid. The plate incubated at 34 °C and the zone of inhibition was observed after two days.

Determination of minimal inhibitory concentration (MIC). *Malassezia fu*rfur CBS1878 was cultured at 34 °C for 3 days in Sabouraud dextrose medium containing 0.1% oleic acid before assay. The antifungal activity of compound **4a** on *M. furfur* was determined by the broth microdilution method (Jorgensen *et al.*, 1999) in 96-well plates. Compound **4a** was prepared as stock solution of 6.7 mg/mL in 90% ethanol. Two-fold dilution of the stock solution was prepared in Sabouraud broth media at concentration from 2000 to 125 μg/mL. Each well was inoculated with 40 μL of *Malassezia furfur* suspension at a density of 10^4 CFU/mL. The micro titer plate was incubated at 34 °C overnight. The 100 μL of cultured solution was spread to Sabouraud dextrose agar plates. Minimal inhibitory concentration is defined as the lowest concentration of compound **4a** that resulted in no visible fungal colony.

Cytotoxicity assay. HepG2 cell line was maintained at 96 well cell culture plate in medium that included 10% FBS with 1% penicillin G/streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator. All experiments were performed in triplicate. Briefly, at 48 hours after various concentrated samples treatment, 10% (vol/vol) of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma) diluted in PBS was added to cell cultures. After 3 hours of incubation, the medi-

um was aspirated and washed with PBS. DMSO (100 μL) was added and shaken gently for 5 minutes and absorbance was measured at 570 nm using a VERSA max microplate reader (Molecular Device Inc.). The mean (±SE) absorbance units obtained from three experiments for each group was compared for statistical significance with t-test, and $P < 0.05$ was considered highly significant.

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