SCREENING FOR MICROBIAL INHIBITORS OF SIGNAL TRANSDUCTION PARTICULARLY THE AKT/GSK-3β PATHWAY

FOO SEK HIN

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Alamat:
Sekolah Sains dan Teknologi
Universiti Malaysia Sabah
Beg Berkunci 2073
88999 Kota Kinabalu
Sabah, MALAYSIA.

(Penyelia: Prof. Dr. Ho Coy Choke)

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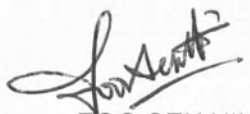

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ABSTRACT

This study aims to screen for microbial inhibitor of GSK-3β using yeast-based screening systems. *gsk-3* null mutant with four yeast GSK-3 homologs (MCK1, MDS1, MRK1 and YOL128C) disrupted, leads to (1) growth defect at 37°C, which is rescued in transformed H10075 (GSK-3β) and H10079 (MCK1), alternatively by inclusion of 1.2M D-sorbitol into the growth medium. (2) Inability to utilize galactose for growth (at 25°C and 37°C), which is suppressed in H10079 (MCK1). Growth of *bul1bul2* mutant is also defective at 37°C, but distinguished in the inability to utilize glycerol. Both phenotypes of *bul1bul2* mutant are suppressed in H10082 (BUL1). Inhibitor of GSK-3β was therefore expected to mimic phenotypes of *gsk-3* null mutant, detected by (A) growth inhibition of H10075 (GSK-3β) and H10079 (MCK1) only at 37°C, confirmed in inhibition of H10084 (MCK1) only at 37°C, and non-inhibition of H10085 (Δmck1). (B) Growth inhibition of H10079 (MCK1) in galactose medium (at 25°C and 37°C) and (C) Non-inhibition of H10082 (BUL1) in glycerol medium (at 25°C and 37°C). Thirty actinomycetes strains previously screened as toxic to yeast were tested on H10075 (GSK-3β). Two strains H7530 and H7667 inhibited H10075 (GSK-3β) only at 37°C, with minimal 1.2M D-sorbitol rescue. Identical inhibition was observed when H7530 and H7667 were tested on H10079 (MCK1) and H10084 (MCK1). In galactose utilization screening, both strains inhibited H10079 (MCK1) in SG-Ura medium, 25°C with stronger inhibition at 37°C. Another 287 actinomycetes strains, isolates from soils of Long Pasia, Lower Segama and Melalap (Crocker Range) were screened on H10075 (GSK-3β) for inhibition at only 37°C, and only two weak inhibitors were found H11329 and H11364. These weak inhibitors were not further studied. Crude extracts of H7530 and H7667 did not inhibit GSK-3β activity *in vitro*. When tested *in vivo* using Western blotting, H7667 inhibited p-GSK-3β (Ser-9) in MCF-7 cells. H7530 had no effect. H10082 (BUL1) and PP1 screenings distinguished these two strains. H7530 inhibited H10082 (BUL1) in glycerol medium at 25°C and 37°C and inhibited H10018 (GLC7) of PP1 screening at 25°C and 37°C. H7667 was negative in the H10082 (BUL1) screen and inhibited H10018 (GLC7) only at 37°C resembled inhibition seen on H10075 (GSK-3β) at 37°C. H7530 and H7667 were purified and bioactive peaks were identified. H7530 (F6) did not reduce p-GSK-3β (Ser-9) in MCF-7 cells. H7667 (F21) inhibited p-GSK-3β (Ser-9) but did not alter p-Akt (Thr-308) indicating H7667 (F21) could probably act directly at the Akt without affecting upstream kinases of Akt. This was substantiated by decrease in p-BAD (Ser-136). Though *in vitro* Akt assay did not detect any significant inhibition, H7667 (F21) may inhibit Akt in a non-ATP competitive manner or by other novel mechanisms. Despite the fact that no inhibitor of GSK-3β was found in this study, an inhibitor, H7667 (F21) that affects the Akt/GSK-3β pathway was identified. This justifies further investigation of H7667 (F21); to determine its specific kinase target and chemical identification of the active compound.

ABSTRAK

MENYARING PERENCAT MIKROBIAL TERHADAP TRANSDUKSI ISYARAT TERUTAMANYA LALUAN AKT/GSK-3β

Kajian ini bertujuan untuk menyaring perencat mikrobial GSK-3β dengan sistem penyaringan yis. Mutan nul gsk-3 yang dibuntukan empat homolognya (MCK1, MDS1, MRK1 dan YOL128C) akan menyebabkan (1) kecacatan pertumbuhan pada 37°C, dipulihkan dalam transforman H10075 (GSK-3β) and H10079 (MCK1), atau dengan penambahan 1.2M D-sorbitol ke dalam medium pertumbuhan. (2) Ketidakupayaan menggunakan galaktos untuk pertumbuhan (25°C and 37°C), dipulihkan dalam H10079 (MCK1). Pertumbuhan H10082 (BUL1) juga dihalang pada 37°C, tetapi dibezakan dengan ketidakupayaan menggunakan gliserol. Kedua-dua fenotip ini dipulihkan dalam H10082 (BUL1). Maka perencat GSK-3β dijangka akan (A) merencatkan H10075 (GSK-3β) and H10079 (MCK1) hanya pada 37°C, dipastikan dengan perencatan H10084 (MCK1) hanya pada 37°C and ketiadaan perencatan pada H10085 (Δmck1). (B) Perencatan H10079 (MCK1) di medium galaktos (25°C and 37°C) dan (C) ketiadaan perencatan pada H10082 (BUL1) di medium gliserol (25°C and 37°C). Tiga puluh strain aktinomiset yang didapati toksik terhadap yis diuji pada H10075 (GSK-3β). Dua strain H7530 dan H7667 didapati merencatkan H10075 (GSK-3β) hanya pada 37°C, dengan pemulihan 1.2M D-sorbitol minima. Perencatan adalah identikal apabila diuji pada H10079 (MCK1) dan H10084 (MCK1). Kedua-dua strain merencat H10079 (MCK1) dalam SG-Ura, dengan perencatan yang lebih kuat pada 37°C. Sebanyak 287 strain aktinomiset dipencilkan daripada tanah Long Pasia, Hilir Segama dan Melalap (Banjaran Crocker) disaring terhadap H10075 (GSK-3β) untuk perencatan hanya pada 37°C, dan hanya dua perencat lemah ditemui, H11329 and H11364. Kedua perencat lemah ini tidak diuji secara lanjut. Ekstrak kasar H7530 dan H7667 tidak merencat aktiviti in vitro GSK-3β. Dalam Western blotting, H7667 merencatkan p-GSK-3β (Ser-9) sel MCF-7, H7530 tidak memberi sebarang kesan. Penyaringan H10082 (BUL1) and PP1 membezakan kedua-dua strain. H7530 merencatkan H10082 (BUL1) di medium gliserol pada 25°C and 37°C, dan merencatkan H10018 (GLC7) pada 25°C dan 37°C. H7667 adalah negatif dalam penyaringan H10082 (BUL1) dan merencatkan H10018 (GLC7) hanya pada 37°C, menyerupai perencatan dalam H10075 (GSK-3β) pada 37°C. Fraksi aktif H7530 and H7667 diasingkan dan peak bioaktif dikenalpasti. H7530 (F6) tidak merencatkan p-GSK-3β (Ser-9). H7667 (F21) merencatkan p-GSK-3β (Ser-9) tetapi tidak mengubah p-Akt (Thr-308), menandakan H7667 (F21) mungkin merencat Akt tanpa mempengaruhi kinase di atas Akt. Ini dibuktikan dalam pengurangan p-BAD (Ser-136). Asai in vitro Akt tidak menunjukkan perencatan yang ketara, yakni H7667 (F21) mungkin merencat Akt bukan secara ATP-kompetitif atau melalui mekanisma lain yang baru. Sungguhpun tiada perencat GSK-3β ditemui, kajian ini menemui H7667 (F21) yang merencatkan laluan Akt/GSK-3β, maka adalah penting untuk mengenalpasti kinase sasaran spesifik serta pengenalan struktur kimia bagi sebatian aktif tersebut.

ABBREVIATIONS

Akt	protein kinase B (also called PKB)
AMPK	AMP-activated protein kinase
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CAMK	calmodulin-dependent protein kinase
CDK	cyclin-dependent protein kinase
CHK	checkpoint kinase
CK	casein kinase
CREB	cAMP-response element binding protein
CSK	C-terminal Src kinase;
DTT	dithiothreitol
DYRK	dual-specificity, tyrosine-phosphorylated and regulated kinase;
EGF	epidermal growth factor
ERK	extracellular-signal-regulated kinase
Exp.	Experiment
FBS	fetal bovine serum
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase-3
hr	hour(s)
HV	humic acid-B vitamins
IC_{50}	concentration giving half maximal inhibition
IDV	Integrated density value
IGF-I	insulin-like growth factor I
ILK	integrin-linked kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
L	litre
LCK	lymphocyte kinase
M	molar
MAPK	mitogen-activated protein kinase
MAPK	mitogen activated protein kinase
MAPKAP-K1	MAPK-activated protein kinase-1

MAPKAP-K2	MAPK-activated protein kinase 2
mg	milligram
min	minute
MKK	MAPK kinase (also called MEK)
MLCK	myosin light chain kinase
mm	millimeter
MNK	MAPK-integrating kinase
M_r	relative molecular mass (unitless)
MSK1	mitogen- and stress-activated protein kinase 1
mTOR	mammalian target of rapamycin
nM	nanomolar
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PHK	phosphorylase kinase
PHLPP	PH domain Leucine-rich repeat protein phosphatase
PI3-kinase	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PMSF	phenylmethylsulfonyl fluoride
PRAK	p38-regulated/activated kinase
PRK	protein kinase C-related protein kinase
ROCK	Rho-dependent protein kinase
S6K1	p70 ribosomal protein S6 kinase
SAPK	stress-activated protein kinase
SAPK2a	stress-activated protein kinase 2a (also called p38)
SAPK2b	stress-activated protein kinase 2b (also called p38b2)
SAPK3	stress-activated protein kinase 3 (also called p38c)
SAPK4	stress-activated protein kinase 4 (also called p38d)
SGK	serum- and glucocorticoid-induced kinase;
t_R	retention time
µg	microgram
µl	microlitre
µM	micromolar

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CHAPTER 1

INTRODUCTION

This study was designed to screen for inhibitor specific to GSK-3β using yeast-based screening systems. Inhibitor in this context refers to small molecule compound synthesized by actinomycetes which acts on GSK-3β of the insulin-activated Akt/GSK-3β pathway. Yeast (*Saccharomyces cerevisiae*) mutants and transformants were tools to develop systems that screen for this inhibitor.

This chapter will therefore review the functional values of some existing small molecule inhibitors, and the progressing discovery which lead to the current interest of this study.

1.1 Small molecule inhibitors of signal transduction: sources and applications

Earlier small molecule inhibitors of signal transduction were predominantly tools in understanding the physiological roles of protein kinases and the molecular mechanisms of protein phosphorylation (Figure 1.1). In the 1980s, research group led by Hidaka utilized synthetic H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine], a selective inhibitor of protein kinase C (PKC) to study the role of PKC while its derivatives H88 and H89 were useful probes in determining physiological function of cAMP-dependent protein kinase (PKA) (Naito *et al.*, 1999). Fungal metabolite, wortmannin and plant flavonoid derivative LY294002 that specifically inhibit PI3K (phosphatidylinositol 3-kinase) are often used to dissect PI3K-mediated pathway.

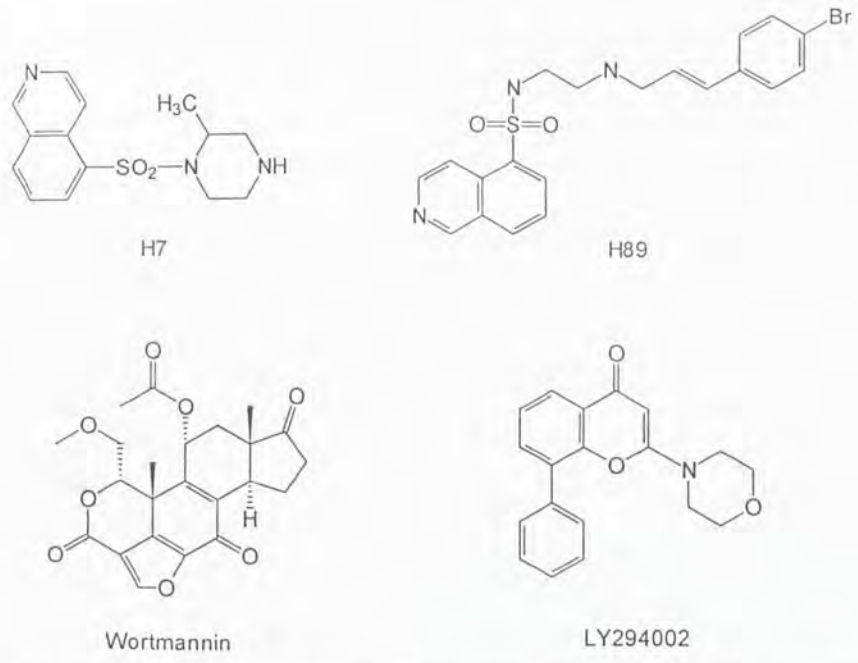

Figure 1.1: Small molecule inhibitors for bioprobing of signaling pathways

Today, apart from being biochemical probes, small molecule inhibitors has become potential therapeutics for molecular diseases such as cancer, diabetes and Alzheimer's, the very important sources of these novel compounds come from nature - terrestrial plants and terrestrial microorganisms. Notable small molecule inhibitors from plants (Figure 1.2) that exhibit antiproliferative properties are (1) flavopiridol, an alkaloid derivative from *Dysoxylum binectarieferum* which inhibits CDK1, CDK2 and CDK4 (Elsayed & Sausville, 2001), (2) indirubin-3'-monoxime, originating from indigo-producing plants (*Indigo naturalis*) that inhibits CDK2 and GSK-3β (Bain *et al.*, 2003; Leclerc *et al.*, 2001) and (3) taxol, isolated from *Taxus brevifolia*, which promotes stabilisation of tubulin polymerization into microtubules, and also activates apoptotic signaling cascades such as JNK,3 Raf-1, and Bcl-2 family members (Broxterman & Georgopapadakou, 2005; MacKeigan *et al.*, 2002).

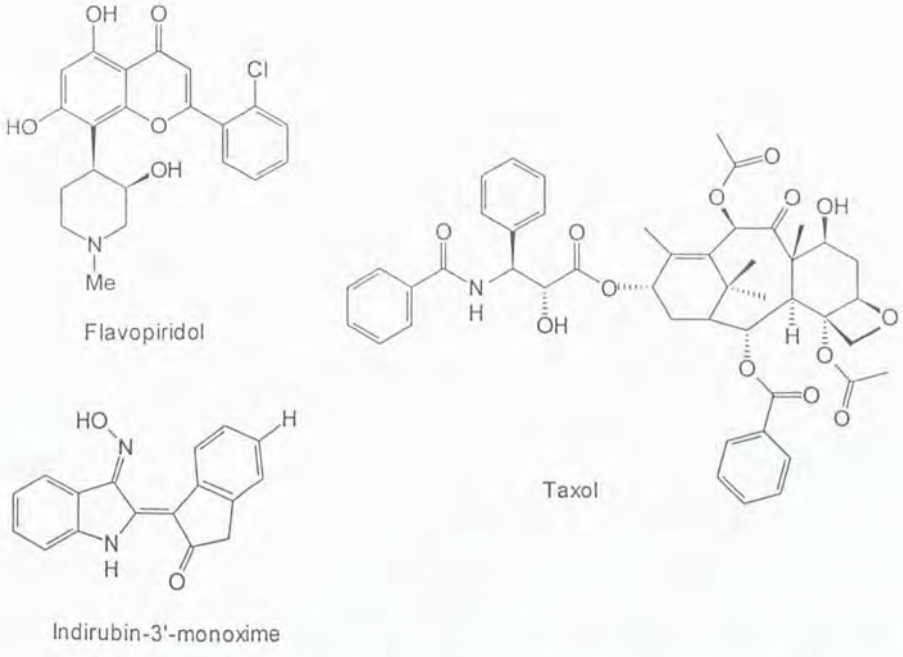

Figure 1.2: Plant-synthesized small molecule anti-proliferative agents

Microbial secondary metabolites represent a large source of compounds endowed with complex structures and potent biological activities. These compounds may not become new drugs, but many are good templates for semisynthetic and total synthetic modification that eventually developed into a drug. Therefore screening of natural products is one of the earliest steps in drug discovery, as to obtain a lead compound.

Active producers of secondary metabolites at the moment are restricted to a few groups of bacterial and eukaryotic microbes. Filamentous actinomycetes, the myxobacteria, the pseudomonads, the cyanobacteria and the eukaryotic filamentous fungi are proven to be producers of many chemically different secondary metabolites, but with the thousands of compounds described in literature, discovering new bioactive metabolites is no easy task (Donadio *et al.*, 2002).

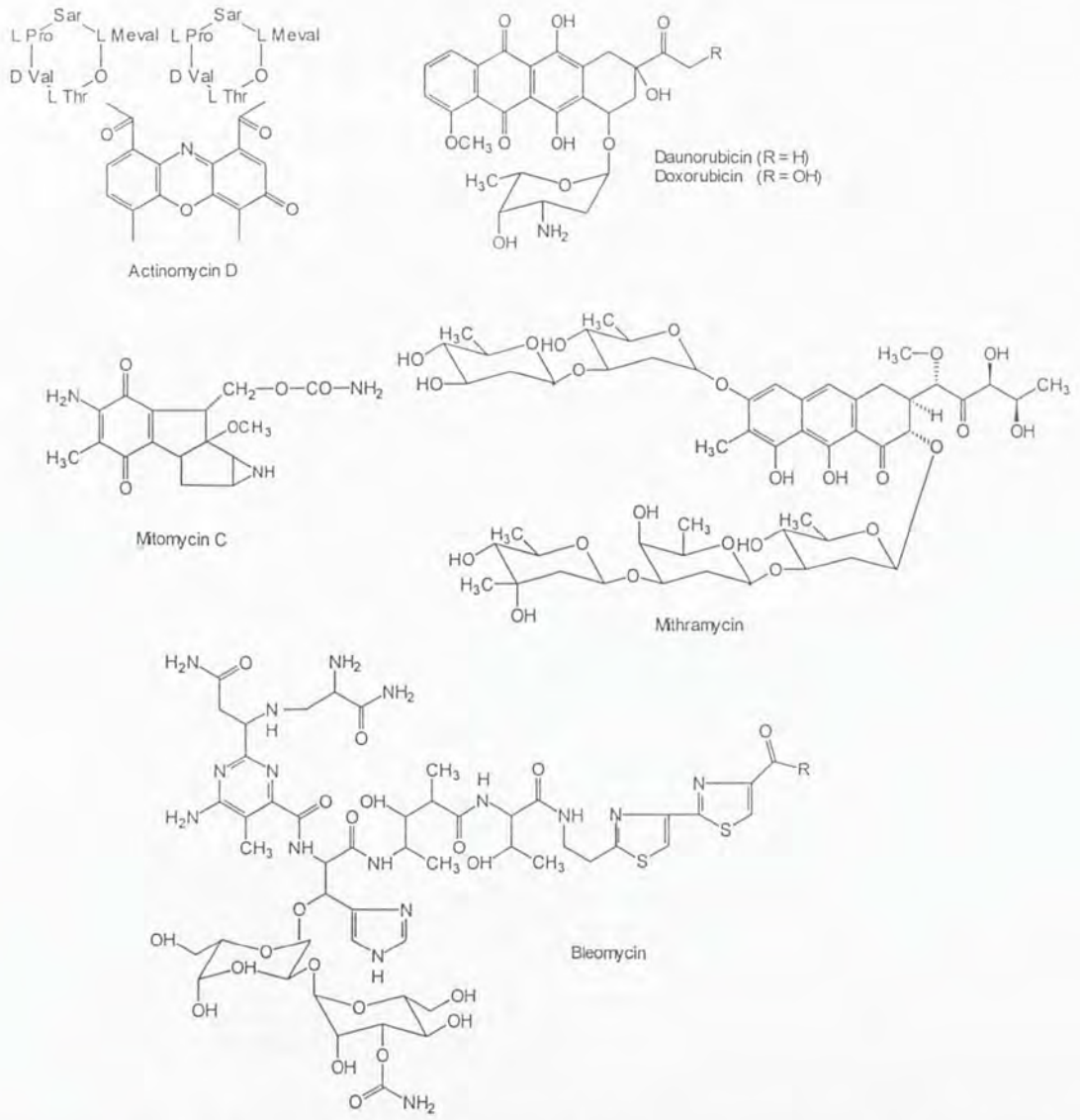

Figure 1.3: Anti-tumor agents produced by actinomycetes with clinical application in cancer chemotherapy

Many of the earlier small molecule anti-tumor agents (Figure 1.3) are synthesized by actinomycetes and these compounds exert cytotoxic action by interfering with DNA function. Actinomycin D and anthracylines (daunorubicin, doxorubicin) intercalate with DNA, mitomycin C is a crosslinking agent, and bleomycin causes DNA strand breakage. The group of compounds that interacts DNA in a non-intercalative way consists of mithramycin, chromomycin and olivomycin (Salas & Méndez, 1998).

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