Signaling Effects of Demethylasterriquinone B1, a Selective Insulin Receptor Modulator

Nicholas J. G. Webster,*[a] Kaapjoo Park,[b] and Michael C. Pirrung[b]

A possible breakthrough in the treatment of diabetes was made with the discovery that a fungal natural product, demethylasterriquinone B1 (DAQ B1), is an orally active, small-molecule mimic of insulin. Subsequent work has shown that the glucose-lowering effects of DAQ B1 are not accompanied by enhanced vascular proliferation, which is a side effect of chronic insulin administration that can lead to arteriosclerosis. Our recent short and modular total synthesis of DAQ B1 could be readily modified to create congeners and afforded ample supplies of the natural product, which permitted intracellular signal transduction of DAQ B1 to be examined. The activities of DAQ B1 and over a dozen related structures were studied for insulin receptor (IR) and insulin receptor substrate-1 phosphorylation. Examination of the effect of DAQ B1 on kinases downstream of the IR in insulin signal transduction showed selective activation of Akt kinase (a metabolic effect) but not of extracellular-regulated kinase (a proliferative effect). The influence of DAQ B1 on gene expression (determined by a microarray study) was also divergent from that of insulin, which activates both proliferative and metabolic pathways. The action of DAQ B1 as a selective insulin receptor modulator can be accounted for by its ability to selectively activate one kinase among the many emanating from insulin receptor autophosphorylation and its reduced effects on gene expression.

KEYWORDS:

gene expression · insulin · kinases · microarray · natural products

Introduction

A long-sought goal in the growth factor receptor field,[1] attainment of small molecule agonists, was realized with the discovery of demethylasterriquinone B1 (DAQ B1), an insulin receptor (IR) activator.[2] Oral administration of DAQ B1 causes lowering of glucose levels in diabetic mice without causing proliferation of vascular smooth muscle,[3] and thus may qualify as the first selective insulin receptor modulator (SIRM). In other animal models, DAQ B1 lowers glucose levels only in the presence of low levels of insulin.[4, 5]

The initial stages of transmembrane signaling that lead to insulin's metabolic effects are shown in Figure 1. A key control molecule is Akt, which supervises glucose transport by GLUT4 as well as glycogen synthase by glycogen synthase kinase-3 (GSK-3). Insulin also affects other signal-transduction molecules, including the Ras proliferative pathways.

We recently completed a brief, modular total synthesis of the natural product DAQ B1.[6] In order to better understand the in vivo actions of this molecule, its metabolic and transcriptional effects were studied in cellular models. In rat fibroblasts that over-express human IR, DAQ B1 shows similar activation of the extracellular-regulated kinase (ERK) from the MAP kinase family to that achieved by insulin, but enhanced activation of the Akt

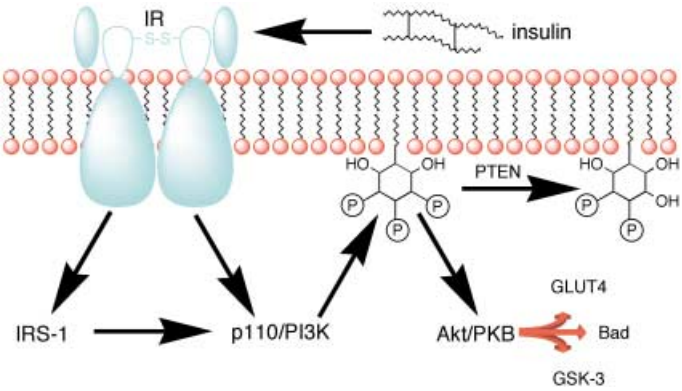


Figure 1. *Insulin signal transduction begins with the binding of insulin to the extracellular domain of the dimeric receptor formed from the smaller α chain and the extracellular domain of the β chain. Phosphorylation of tyrosine residues in the intracellular domain of the β chains occurs, which permits phosphorylation of insulin receptor substrates such as IRS-1. It is believed that DAQ B1 directly promotes phosphorylation of the intracellular tyrosine kinase domain of the IR. The phosphotyrosine residues in the IR or IRS-1 can recruit phosphatidylinositol-3-kinase (PI3K) to the cell membrane through adapter protein interactions with SH2 domains. Production of phosphatidylinositol-3,4,5-tris-phosphate activates Akt for phosphorylation of GLUT4 for glucose transport and GSK-3 for glycogen synthesis. Phosphatases such as PTEN inhibit some insulin (and other proliferative) actions by dephosphorylating lipid phosphate groups. Protein tyrosine phosphatase 1B (PTP1B) can inhibit some insulin actions by dephosphorylating IR and IRS-1. Bad = pro-apoptotic BCL-2 family member.*

[a] *Prof. N. J. G. Webster*
Department of Medicine 0673
University of California-San Diego, 9500 Gilman Drive
La Jolla, California 92093 (USA)
and
San Diego Veterans Healthcare System, CA 92161 (USA)
Fax: (+1) 858534-6653
E-mail: nwebster@ucsd.edu

[b] *K. Park, Prof. M. C. Pirrung*
Department of Chemistry
Levine Science Research Center
Duke University, Durham, North Carolina 27708 (USA)

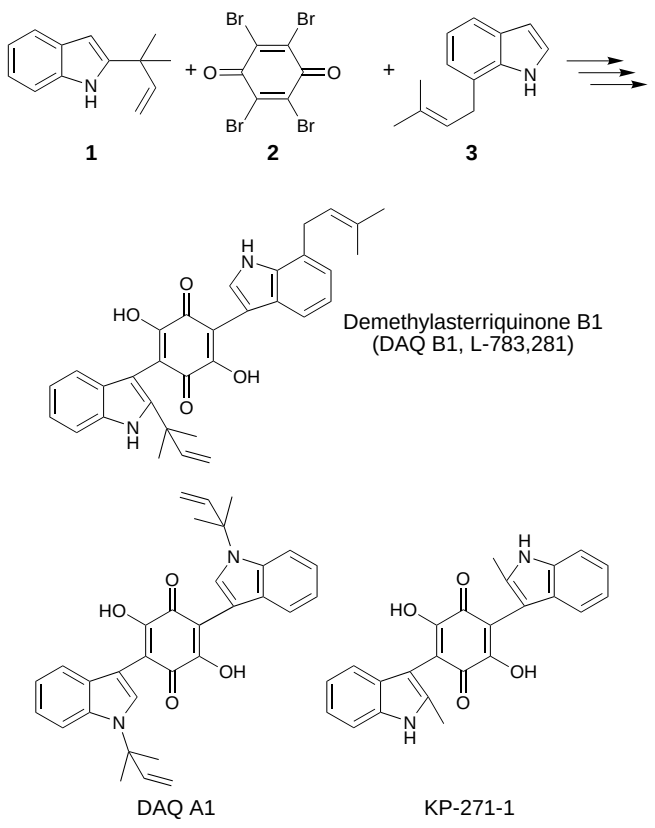
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 DOI: 10.1002/cbic.200200468

kinase. In adipocytes, DAQ B1 promotes glucose transport. In hepatoma cells, insulin induces expression of cell-cycle genes, while DAQ B1 up-regulates metabolic gene expression. The SIRM properties of DAQ B1 may thus be attributed to its enhancement of Akt phosphorylation relative to ERK, and its failure to induce proliferative gene expression.

Results and Discussion

A first-generation, modular total synthesis of DAQ B1 (Scheme 1) was achieved in three steps and 41% overall yield by adaptation of a synthesis of symmetrical bis-indolylquinones[7] to produce the known modules 2-isoprenylindole (**1**) and 7-prenylindole (**3**). This synthesis provides the ability to prepare targets bearing two different indoles, an improvement on earlier synthetic methods for bis-indoloquinones. This feature is attractive because unsymmetrical bis-indoloquinone congeners of DAQ B1 can be more structurally diverse than symmetrical ones, and it is unsymmetrical asterriquinones which act upon the IR (see below).



Scheme 1. *Modular synthesis of demethylasterriquinone B1.*

The action of synthetic DAQ B1 on insulin signal transduction was studied in hIRcB rat fibroblasts expressing approximately 3×10^6 human insulin receptors (Figure 2).[8] Insulin causes a dose-dependent increase in tyrosine phosphorylation of the IR β subunit, as well as of the endogenous substrates IRS-1 and the three isoforms of the Src homology and collagen protein (Shc; p44, p52, and p66). DAQ B1 also causes an increase in tyrosine

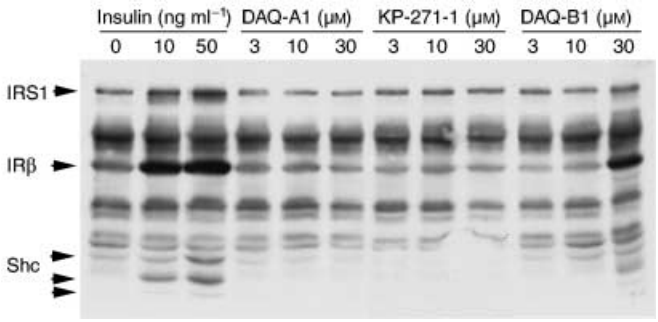


Figure 2. *Stimulation of the insulin receptor by DAQ-B1. hIRcB cells were serum starved for 72 h in 12-well plates, then stimulated with vehicle and insulin (10 or 50 ng mL^{-1}) or asterriquinone DAQ-B1, DAQ-A1, or KP-271 (3, 10, and 30 μM) for 5 min at 37°C. Whole cell lysates were immunoblotted with an anti-phosphotyrosine antibody (RC20H). Phosphorylated proteins were visualized by enhanced chemiluminescent detection.*

phosphorylation at a concentration of 30 μM equivalent to that observed at an insulin concentration of 10 ng mL^{-1}, which shows that the synthetic compound has an activity concordant with that reported for the natural product. In CHO.IR cells,[1] the EC_{50} value for natural DAQ B1 was reported to be 3 μM. DAQ B1 stimulation of phosphorylation of IRS-1 was also observed. Two symmetrical asterriquinone analogues (KP-271-1 and DAQ A1[6]) have no effect on IR or IRS-1 phosphorylation in this assay; the symmetrical DAQ B4 was earlier reported to be inactive.[1] We also examined 14 mono-indolylquinones.[9] None of these are able to stimulate the IR tyrosine kinase.

All three bis-indolylquinones, DAQ B1, DAQ A1, and KP-271-1, were examined at 30 μM in a glioma cell line that over-expresses insulin-like growth factor I receptors (IGF-I R) to test for activation of this highly homologous kinase domain. None were able to activate the IGF-I R (data not shown), a result in agreement with the proposal that the asterriquinones are specific insulin receptor activators. DAQ B1 also activated two kinases downstream in the insulin signal transduction pathway in hIRcB cells (Figure 3A). Insulin activated the IR, PI-3 Kinase, Akt, and ERK1 and 2 with similar potencies (Figure 3B; $EC_{50} = 2-3$ ng mL^{-1}) but DAQ B1 was a more potent activator of Akt than of IR, PI-3 Kinase, or ERK1 or 2 (Figure 3C; $EC_{50} = 6$ μM, 20 μM, 20 μM, and 11 μM, respectively). Hence, DAQ B1 is a more selective agonist for the PI-3 Kinase/Akt pathway than insulin.

The action of synthetic DAQ B1 in promoting the metabolic effect of glucose transport was examined in 3T3 L1 adipocytes in culture. DAQ B1 caused a dose-dependent increase in glucose transport (Figure 4). The maximal effect, however, was only 50% that of insulin. DAQ B1 does not inhibit insulin-stimulated transport at the dose required for maximal effect, which rules out both direct competition for transport and an inhibitory effect of the compound on signaling.

To determine whether insulin and DAQ B1 stimulation lead to different cellular phenotypes, we examined the transcriptional effects of insulin versus DAQ B1 in the human HepG2 hepatoma cell line.[10] Gene expression in DAQ B1 or insulin-treated cells compared to basal cells was analyzed in duplicate by using HG-U95A microarrays (> 12000 genes) and the results were analyzed

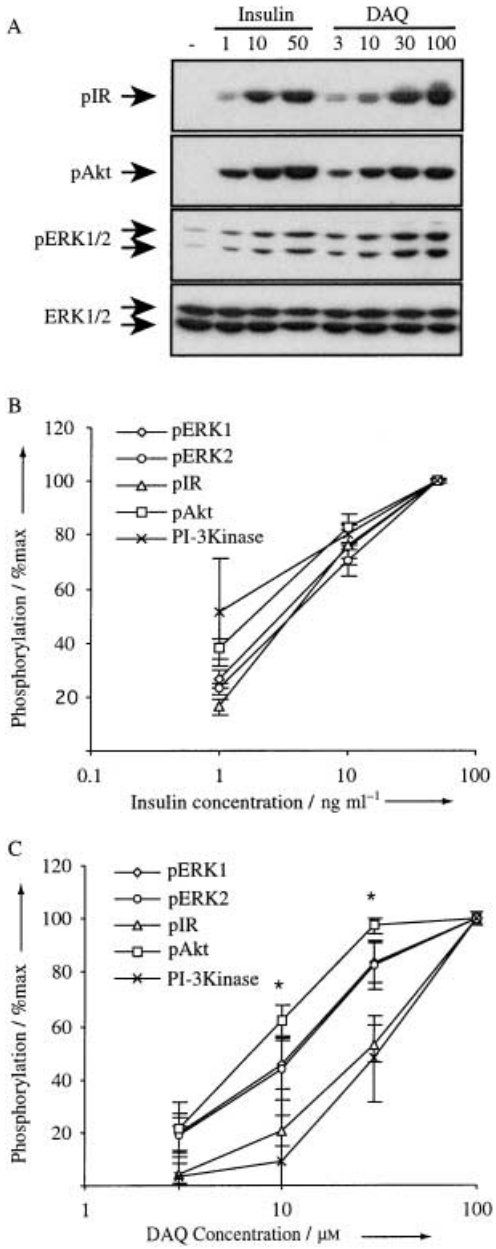


Figure 3. *The effect of DAQ on signaling compared to that of insulin. Serum-starved hIRcB cells were stimulated with increasing concentrations of insulin (1, 10, or 50 ng mL^{-1}) or DAQ-B1 (3, 10, 30, or 100 μM) for 10 min. Panel A: whole cell lysates were immunoblotted with anti-phospho-Tyr1162/Tyr1163 IR (top panel), anti-phospho-Ser473 Akt (second panel), anti-phospho-Thr202/Tyr204 ERK (third panel), or anti-ERK1/2 antibodies (bottom panel). Panel B: insulin-stimulated phosphorylation was quantified by densitometry of autoradiographs. The graph shows the mean change in normalized signal intensity (+SEM) of three experiments and data is expressed as percent maximal phosphorylation. Panel C: DAQ-stimulated phosphorylation was quantified by densitometry of autoradiographs. The graph shows the mean +SEM of five experiments and data is expressed as percent maximal phosphorylation. Asterisks indicate statistical significance (Probability of wrongly rejecting the null hypothesis, $P < 0.05$) of phosphorylation of Akt versus that of the insulin receptor.*

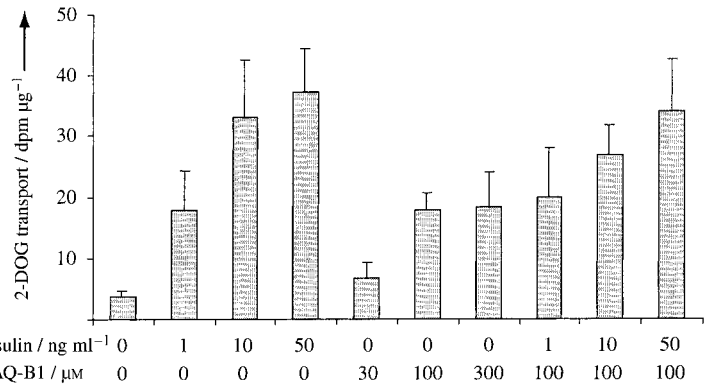


Figure 4. *Effect of DAQ-B1 on glucose transport compared to that of insulin. 3T3L1 adipocytes were stimulated with increasing concentrations of insulin (1, 10, or 50 ng mL^{-1}) or DAQ-B1 (30 or 100 μM) or both for 30 min. Glucose transport was measured by monitoring ^{3}H-2-deoxyglucose (^{3}H-2-DOG) levels for 10 min.*

withthe GeneSpring Software (Ver. 4.0.4). Of the 12000 genes represented on the array, 2328 were present on at least two chips. Of these genes, the expression of 309 was more than doubled and that of 215 decreased by more than half either by insulin or by DAQ B1 treatment. Of the 309 genes that were up-regulated, the expression of 76 genes was increased by both insulin and DAQ B1, that of 37 genes was increased by DAQ B1 alone, and expression of 196 genes was increased by insulin alone. Of the 215 repressed genes, expression of 17 genes was decreased by both insulin and DAQ B1, that of 157 genes was decreased by DAQ B1 alone, and 41 genes showed decreased expression in the presence of insulin alone. Transcript levels varying by more than twofold from basal levels are summarized in tables in the Supporting Information. The expression of 28 genes (22 known, 6 unknown) was altered more than 10-fold by treatment with DAQ B1. The expression of 31 genes (21 known, 10 unknown) was altered by more than 10-fold by insulin. Known genes from these groups whose expression was significantly altered are listed in Table 1. The mean change in normalized signal intensity is listed as well as the fold change. The validity of the DNA array approach can be seen by examining known insulin-regulated genes. IGFBP-1 and G6PT expression decreased 4.8- and 9.3-fold, respectively. The immediate early gene CL100 and the PAI-1 gene are induced 3.6-fold and more than 100-fold, respectively.

The identification of a set of genes that are regulated by DAQ B1 but not insulin confirms that DAQ B1 has effects on cells that are not IR-mediated. This result might be expected as DAQ B1 has cytotoxic effects on cells that are not shared with insulin. Whether these genes are activated in response to chemical stress caused by DAQ B1 is not known, but the induction of the gene for the heat shock transcription factor Hsf4 strongly suggests this is the case. The identification of a set of genes that was regulated by insulin but not altered by DAQ B1 is more significant and suggests that activation of the IR by the two agonists is not equivalent. As all insulin effects at this concentration are mediated by the insulin receptor, activation of the receptor by insulin must engage different signaling pathways than activation by DAQ B1. Interestingly, DAQ B1 fails to induce the Id1, c-myc, and Bcl-X genes. These genes encode proteins that are transcriptional regulators of the cell cycle and inhibitors of apoptosis. We can speculate that the lack of proliferation and cytotoxicity of DAQ B1 is due to failure to express these or similar genes. The

Table 1. *Effects of IR activators on gene expression in Hep2G cells.*[a]

Gene	Accession number	Mean Δ intensity with DAQ	SEM	$P < 0.05$[b]	Mean Δ intensity with insulin	SEM	$P < 0.05$	Fold induction with DAQ[c]	Fold induction with insulin[c]
Genes whose expression was increased by both insulin and DAQ-B1									
PM1	X51804	1.19	0.26	*	1.29	0.21	*	> 100	> 100
SUR1	L78207	1.22	0.24	*	1.19	0.15	*	> 100	> 100
ISOT	U47927	1.16	0.42	*	0.92	0.04	*	6.92	5.72
CI100	X68277	1.02	0.36	*	0.85	0.15	*	4.14	3.60
MTVR	AF052151	0.66	0.05	*	1.19	0.13	*	2.96	4.55
PSMD9	AI347155	0.67	0.04	*	1.19	0.13	*	2.99	4.55
RCCP1	X99720	0.57	0.17	*	2.04	0.31	*	2.41	6.10
FEN1	AC004770	0.30	0.05	*	0.73	0.13	*	1.42	2.04
Genes whose expression was decreased by both insulin and DAQ-B1									
ABC14	AF070598	− 1.98	0.34	*	− 2.32	0.55	*	0.15	< 0.01
HSTF1	M64673	− 2.22	0.50	*	− 1.50	0.71	*	0.18	0.45
USF2	Y07661	− 1.99	0.66	*	− 1.59	0.38	*	0.25	0.40
SURF1	Z35093	− 0.91	0.07	*	− 0.97	0.05	*	0.52	0.48
B4-2	U03105	− 0.91	0.30	*	− 1.40	0.40	*	0.50	0.22
IGFBP1	M74587	− 0.38	0.01	*	− 1.10	0.03	*	0.72	0.21
IGFBP4	M62403	− 0.93	0.02	*	− 0.39	0.61	*	0.48	0.78
HNPCC6	D50683	− 0.94	0.07	*	− 0.83	0.10	*	0.48	0.54
PBP	L33243	− 1.49	0.04	*	− 1.36	0.14	*	0.37	0.42
Genes whose expression was decreased by DAQ-B1 but increased by insulin									
SC35	X75755	− 0.99	0.22	*	1.21	0.12	*	< 0.01	2.22
SRF	J03161	− 0.56	0.00	*	0.70	0.15	*	0.45	1.70
FAS	U29344	− 0.40	0.13	*	0.92	0.05	*	0.61	1.92
PP2A R65	J02902	− 0.40	0.04	*	0.43	0.08	*	0.60	1.43
Genes whose expression was increased by insulin alone									
RPA39	AF008442	0.00	0.00		5.98	0.84	*	n/a[d]	> 100
PAI 1	J03764	1.72	1.71		2.23	0.54	*	> 100	> 100
GADD45	M60974	0.00	0.00		5.60	1.29	*	n/a[d]	> 100
clk3	L29211	0.00	0.00		1.48	0.29	*	n/a[d]	> 100
Ets2	J04102	0.34	0.34		1.39	0.22	*	> 100	> 100
Bclx	Z23115	0.00	0.00		26.70	8.37	*	n/a[d]	> 100
PDK1	AF017995	0.00	0.00		1.27	0.01	*	n/a[d]	> 100
TNFR2 − RP	L04270	0.35	0.35		1.79	0.13	*	> 100	> 100
Id1	X77956	0.00	0.00		9.78	1.90	*	n/a[d]	> 100
RCC1	X12654	0.00	0.00		2.53	0.53	*	n/a[d]	> 100
EIF4F	D12686	0.00	0.00		2.39	0.35	*	n/a[d]	> 100
RHOH6	M12174	0.71	0.71		4.44	0.94	*	> 100	> 100
LIS1	L13385	0.75	0.74		1.26	0.13	*	> 100	> 100
BET 3	AJ224335	0.00	0.00		1.66	0.38	*	n/a[d]	> 100
TRBP2	U08998	1.11	0.57		1.45	0.07	*	25.67	33.22
SREBP1	U00968	− 0.20	0.31		2.50	0.16	*	0.61	5.90
IEX1	S81914	− 0.09	0.24		3.50	0.75	*	0.90	4.93
TCTA	L41143	− 0.11	0.50		2.25	0.58	*	0.82	4.69
FLRG	U76702	− 0.64	0.64		2.02	0.43	*	< 0.01	4.15
PE-2	U15655	− 0.55	0.55		1.47	0.50	*	< 0.01	3.66
MYC	V00568	0.06	0.33		1.97	0.38	*	1.08	3.59
FBX-1	Z36714	− 0.20	0.33		1.15	0.34	*	0.62	3.16
ETR101	M62831	0.02	0.12		1.70	0.43	*	1.02	3.02
NOP120	X55504	− 0.61	0.35		1.71	0.55	*	0.37	2.78
eIF3	U78525	0.08	0.38		0.99	0.22	*	1.11	2.41
G19P1	J03075	0.32	0.23		0.77	0.04	*	1.51	2.25
VEGFA	AF024710	− 0.67	0.32		1.18	0.15	*	0.32	2.20
beta 2	X02344	0.17	0.17		0.77	0.26	*	1.23	2.06
Genes whose expression was decreased by insulin alone									
G6PT	U01120	− 0.06	0.08		− 1.28	0.42	*	0.95	< 0.01
DEPP	AB022718	− 0.35	0.22		− 1.54	0.51	*	0.77	< 0.01
CLIC4	AL080061	− 2.15	0.82		− 2.38	0.59	*	0.28	0.20
HSPA1L	M11717	0.97	1.22		− 0.97	0.24	*	1.77	0.23
MEK1	L11284	− 1.32	1.46		− 1.57	0.53	*	0.43	0.32
RIG	J02984	0.10	0.14		− 0.73	0.02	*	1.08	0.39
A6RP	Y17169	− 0.48	0.28		− 0.92	0.16	*	0.71	0.45
Genes whose expression was increased by DAQ-B1 alone									
CDC34	L22005	0.78	0.09	*	1.63	1.62		> 100	> 100
Hsf4	D87673	1.82	0.27	*	0.00	0.00		> 100	n/a[d]
SPO	U39573	2.54	0.02	*	0.00	0.00		> 100	n/a[d]

Table 1. *(Continued)*

Gene	Accession number	Mean Δ intensity with DAQ	SEM	$P < 0.05$[b]	Mean Δ intensity with insulin	SEM	$P < 0.05$	Fold induction with DAQ[c]	Fold induction with insulin[c]
BCSG1	AF044311	150.50	49.30	*	0.00	0.00		> 100	n/a[d]
HSP86	X15183	1.27	0.20	*	0.57	0.56		> 100	> 100
NCAM	M22092	0.22	0.21	*	0.88	0.88		> 100	> 100
c-fos	V01512	1.45	0.16	*	0.92	0.57		8.84	5.95
SAP18	U96915	1.30	0.40	*	0.83	0.37		5.80	4.06
klf6	AF001461	1.20	0.19	*	− 0.33	0.32		4.69	< 0.01
ANG	M11567	1.50	0.34	*	0.39	0.25		3.46	1.64
PCK1	L12760	0.85	0.30	*	0.58	0.47		2.75	2.19
PAP39	D61391	0.90	0.30	*	0.45	0.37		2.38	1.68
Genes whose expression was decreased by DAQ-B1 alone									
PRK1	U33053	− 1.15	0.11	*	0.01	0.19		< 0.01	1.00
ERK3	X80692	− 1.71	0.09	*	− 0.80	0.91		< 0.01	0.54
DDAHII	AJ012008	− 1.18	0.18	*	− 0.26	0.17		0.14	0.81
CDH14	U59325	− 0.86	0.18	*	1.08	0.52		0.17	2.04
AMP	Y00486	− 0.87	0.02	*	− 0.25	0.26		0.38	0.82
CHES1	U68723	− 0.84	0.01	*	− 0.79	0.69		0.43	0.47
Rac-alpha	M63167	− 0.84	0.28	*	− 0.99	0.56		0.46	0.36
ERRalpha	L38487	− 0.52	0.05	*	0.26	0.13		0.48	1.26
PP4	X70218	− 0.63	0.03	*	− 0.23	0.11		0.49	0.81

[a] Data are presented as change in normalized signal intensity from mean basal value (mean + SEM). [b] Asterisks indicate that the mean change in normalized signal intensity is statistically different from zero ($P < 0.05$). [c] The fold induction for genes not expressed in the basal state is arbitrarily set at more than 100. The fold induction for genes not expressed in the stimulated state but expressed in the basal state is set as less than 0.01. [d] n/a indicates that the gene was not expressed in either basal or stimulated state.

microarray experiment also offers a potential explanation for the enhanced Akt activation seen with DAQ-B1. Akt is dephosphorylated by the phosphatase PP2A.[11] The R65 subunit of PP2A is up-regulated by insulin but down-regulated by DAQ B1. The up-regulation by insulin may be part of a negative feedback loop that serves to terminate Akt signaling, so down-regulation by DAQ B1 may enhance and prolong Akt activation.

The cellular effects of DAQ B1 on gene expression and Akt/ERK phosphorylation may provide a clue to its selective action on vascular smooth muscle, where it is known to have metabolic but not proliferative effects. Another known property of the asterriquinones may provide a mechanism for this differential action. A number of asterriquinone derivatives antagonize the interaction of phosphorylated epidermal growth factor receptor with its adaptor protein, Grb2, and thereby inhibit cellular proliferation.[12–15] Likewise, DAQ B1 may prevent coupling of Grb2 to the insulin mitogenic pathway while simultaneously allowing the IR to promote glucose transport. This antagonism provides a molecular explanation for the action of the first selective insulin receptor modulator.

Conclusion

This work establishes the ability of a small molecule that already has very interesting and powerful effects on insulin signal transduction to act with specificity on multiple elements of a very complex intracellular signal transduction process. The results pose the further puzzle of how closely related molecules can inhibit phosphorylation-based signaling in one instance and enhance it in another. The ability to simultaneously up-regulate one segment and down-regulate another segment of a signal transduction cascade may have real value in modifying biological response functions, a feature that might even be contained within a single molecule such as DAQ B1. This work also adds to the quite sparse data on the transcriptional effects of insulin.[16]

Methods

Immunoblotting: Anti-phosphoSer473 Akt, anti-phosphoERK Thr202/Tyr204, and ERK1/2 antibodies were obtained from Cell Signaling Technologies (Beverly, MA), catalogue nos. 9271, 9101, and 9102. Anti-phosphoIR (Y1162/63) antibodies were from BioSource (Camarillo, CA) catalogue nos. 44 – 804. Anti-IRS-1 antibodies were from Upstate (Lake placid, NY), catalogue nos. 06-248. Anti-phosphotyrosine antibodies RC20H are from Transduction Labs (Lexington, KY), catalogue no. E120H. hIRcB cells were grown in DME/F12 medium supplemented with fetal calf serum (FCS; 10%), glucose (25 mM), glutamax (2 mM), and gentamicin at 37 °C in a 10% CO_2 environment. Cells were serum starved for 72 h in 12-well plates then stimulated by treatment with increasing concentrations of insulin or asterriquinones DAQ A1, KP-271-1, or DAQ B1 in KRP-HEPES for 5 or 10 min at 37 °C. The cells were washed with ice-cold phosphate-buffered saline and solubilized in 2 × sodium dodecyl sulfate (SDS)-sample buffer containing sodium orthovanadate (2 mM) and sodium fluoride (200 mM). The proteins were denatured by boiling for 5 min, then separated by electrophoresis on 7.5% SDS-PAGE and transferred to polyvinylidine fluoride membranes. The filter was blocked with bovine serum albumin (BSA; 3%) in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 30 min and incubated with the anti-phosphotyrosine Fab – HRP conjugate RC20H, anti-phospho-Tyr1162/Tyr1163-IR, anti-phospho-Ser473-Akt, anti-phospho-Thr202/Tyr204-ERK, or anti-ERK1/2 antibodies at a dilution of 1:1000 in blocking buffer for 2 h. The filters were washed with T-TBS for 30 min

and incubated with horseradish-peroxidase-conjugated secondary antibodies. Enhanced chemiluminescent detection was used to visualize the tyrosine-phosphorylated proteins. The percentage activation and errors in these measurements are summarized in Table 2.

Evaluation of IRS-1-associated PI-3 kinase activity: In vitro phosphorylation of phosphatidylinositol was carried out in IRS-1 immunoprecipitates. hIRcB fibroblasts were serum starved for 72 h then incubated in the presence of increasing doses of insulin or DAQ-B1 in KRP-HEPES (Krebs-Ringer phosphate 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) for 10 min. After lysis in HEPES (50 mM), NaCl (150 mM), ethylenediaminetetraacetate (10 mM), Triton X-100 (1%), sodium fluoride (200 mM), glycerol (10%), orthovanadate (4 mM), aprotinin (800 KIU mL^{-1}), and benzamidine (15 mM), cells were subjected to immunoprecipitation by treatment with an anti-IRS-1 antibody overnight at 4 °C. Immune complexes were precipitated from the supernatant with protein A-Sepharose and washed extensively in lysis buffer. Complexes were incubated with phosphatidylinositol and [γ^{32}P] adenosine triphosphate (3000 Ci $mmol^{-1}$) for 10 min at room temperature. Reactions were stopped by treatment with HCl (20 μL, 8 N) and $CHCl_3$: methanol (1:1, 160 μL) and centrifuged. The lower organic phase was removed and applied to potassium oxalate (1.3%) coated silica gel TLC plates (Merck). Lipid products were resolved by ascending chromatography in $CHCl_3$/MeOH/NH_4OH/H_2O (120:94:4:22.6). The TLC plates were air dried and the labeled phosphatidylinositol visualized by autoradiography and quantified by densitometry.

Evaluation of glucose transport in adipocytes: Merck originally reported that the compound DAQ B1 stimulated glucose transport in primary adipocytes and intact mouse soleus muscle. We tested whether synthetic DAQ B1 could stimulate transport in 3T3L1 adipocytes. 3T3L1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with glucose (25 mM), penicillin (50 units mL^{-1}), streptomycin (50 μg mL^{-1}), and 10% FCS in a 10% CO_2 environment. Cells were then differentiated 2 days post confluence by the addition of complete medium containing isobutylmethylxanthine (500 μM), dexamethasone (25 μM), and insulin (4 μg mL^{-1}). After 2 days, cells were grown in media containing only insulin for a further 3 days. Differentiated adipocytes were re-plated into appropriate dishes. The medium was then changed to regular DMEM glucose with 10% FCS and replaced every 2 days until the cells were well differentiated (day 10) with many lipid droplets. Cells in 12-well plates were rinsed three times with phosphate-buffered saline at 23 °C and preincubated with KRP-HEPES buffer (0.5 mL) containing 0.5% fatty-acid-free BSA in the presence of increasing concentrations of insulin or DAQ B1 (30 μM) at 37 °C for 30 min. The transport reaction was initiated by adding KRP-HEPES buffer (50 μL) containing 2-deoxyglucose (1.1 mM; 50 μM final) with [^{3}H]2-deoxyglucose (0.2 μCi/well). After a 10-min incubation period, cells were washed three times with ice-cold phosphate-buffered saline containing phloretin (100 mM), solubilized in 1% SDS, 0.1% NaOH, neutralized, and counted. Nonspecific uptake was measured in the presence of cytochalasin B (25 μM).

Gene expression profiling in HepG2 hepatoma cells: To investigate whether DAQ activates the IR in exactly the same manner as insulin we undertook gene expression profiling studies. We chose HepG2 cells as insulin has major transcriptional effects in the liver that repress gluconeogenic genes and stimulate glycolysis and fatty acid synthesis. In vivo studies have shown that the effect of insulin on the liver can be seen between 90 min and 4 h after administration, so we chose 4 h as a single time point. Cells were plated in duplicate 10-cm dishes and starved of serum for 48 h prior to stimulation with insulin (10 ng mL^{-1}) or DAQ B1 (30 μM) for 4 h. These concentrations of agonists were chosen as they gave equivalent activation of the IR in hIRcB cells. Total RNA was harvested and purified on RNeasy spin columns (Qiagen, Valencia, CA). The mRNA was quantified and its integrity checked by agarose gel electrophoresis. mRNA (10 μg) from basal cells, or cells treated with insulin or DAQ B1 was given to the UCSD Genechip Core facility for analysis on Affymetrix human U95A gene chips. Duplicates were run with independently isolated RNA for each set of conditions. Data was analyzed by using the Affymetrix Microarray Suite and Silicon Genetics GeneSpring Ver. 4 software programs. Genes were considered expressed if they were scored as present on two of the six chips. Hybridization to the chips was normalized both between chips and within each chip to eliminate local variations in hybridization. The duplicates were then averaged with the GeneSpring program and the three groups of results compared.

Supplementary information: Procedures for preparation of KP-271 – 1, structures of indoloquinones studied, and tables of gene expression effects.

Financial support was provided by the American Diabetes Association and the Diabetes Action Research and Education Foundation. We acknowledge the outstanding technical assistance of Darrel A. Austin.

[1] C. McInnes, B. D. Sykes, *Biopolymers* **1997**, *43*, 339 – 366.
[2] B. Zhang, G. Salituro, D. Szalkowski, Z. Li, Y. Zhang, I. Royo, D. Vilella, M. T. Diez, F. Pelaez, C. Ruby, R. L. Kendall, X. Mao, P. Griffin, J. Calaycay, J. R. Zierath, J. V. Heck, R. G. Smith, D. E. Moller, *Science* **1999**, *284*, 974 – 977.
[3] M. A. Weber, A. Lidor, S. Arora, G. M. Salituro, B. B. Zhang, A. N. Sidawy, *J. Vasc. Surg.* **2000**, *32*, 1118 – 1126.
[4] S. A. Qureshi, V. Ding, Z. Li, D. Szalkowski, D. E. Biazzo-Ashnault, D. Xie, R. Saperstein, E. Brady, S. Huskey, X. Shen, K. Liu, L. Xu, G. M. Salituro, J. V. Heck, D. E. Moller, A. B. Jones, B. B. Zhang, *J. Biol. Chem.* **2000**, *275*, 36590 – 36595.

Table 2. *Percentage activation of the tyrosine-phosphorylated proteins visualized by immunoblotting.*

	pERK1	pERK2	pIR	pAkt	PI-3K	SEM ERK1	SEM ERK2	SEM IR	SEM Akt	SEM PI-3K
DAQ-B1 [μM]										
3	19.65	19.08	4.53	21.63	3.51	6.37	8.18	3.949	9.52	18.88
10	45.81	43.69	20.67	61.78	9.50	9.327	11.28	6.026	5.75	26.99
30	83.51	82.75	53.34	97.40	47.68	7.76	9.03	6.658	2.68	16.22
100	100	100	100	100	100	0	0	0	0	2.68
Insulin [ng mL^{-1}]										
1	26.77	22.78	16.18	37.86	51.33	2.38	3.18	2.57	3.55	19.83
10	75.71	69.94	76.20	82.70	80.51	1.56	1.52	11.71	0.51	4.13
50	100	100	100	100	100	0	0	0	0	1.01

[5] K. Liu, L. Xu, D. Szalkowski, Z. Li, V. Ding, G. Kwei, S. Huskey, D. E. Moller, J. V. Heck, B. B. Zhang, A. B. Jones, *J. Med. Chem*. **2000**, *43*, 3487 – 3494.
[6] M. C. Pirrung, Z. Li, K. Park, J. Zhu, *J. Org. Chem.* **2002**, *67*, 7919.
[7] G. D. Harris, A. Nguyen, H. App, P. Hirth, G. McMahon, C. Tang, *Org. Lett*. **1999**, *1*, 431 – 434.
[8] G. C. Rolband, J. F. Williams, N. J. Webster, D. Hsu, J. M. Olefsky, *Biochemistry* **1993**, *32*, 13 545 – 13 550.
[9] M. C. Pirrung, L. Deng, Z. Li, K. Park, *J. Org. Chem.* **2002**, *67*, 8374.
[10] A. Kosaki, T. S. Pillay, L. Xu, N. J. G. Webster, *J. Biol. Chem*. **1995**, *270*, 20 816 – 20 823.
[11] D. Chen, R. V. Fucini, A. L. Olson, B. A. Hemmings, J. E. Pessin, *Mol. Cell. Biol*. **1999**, *19*, 4684 – 4694.
[12] D. Harris, A. Nguyen, L. Strawn, A. Fong, H. App, T. Le, B. Sutton, P. C. Tang, **1998**, *215th ACS National Meeting*, Dallas, MEDI-163.
[13] P. C. Tang, G. McMahon, G. D. Harris (Sugen), PCT Int. Appl. WO 9640115 A1 19961219, **1996** [*Chem. Abstr.* **1997**, 126: 139873].
[14] P. C. Tang, G. McMahon, G. D. Harris, Jr., K. Lipson (Sugen), PCT Int. Appl. WO 0121589 A2 20010329, **2001** [*Chem. Abstr.* **2001**, 134: 252259].
[15] P. C. Tang, G. McMahon, G. D. Harris, (Sugen), US 5780496, **1998** [*Chem. Abstr.* **1998**, 129: 90451].
[16] C. Mulligan, J. Rochford, G. Denyer, R. Stephens, G. Yeo, T. Freeman, K. Siddle, S. O'Rahilly, *J. Biol. Chem*. **2002**, *277*, 42 480 – 42 487.

Received: August 6, 2002
Revised: February 24, 2002 [F 468]

