

ATP non-competitive IGF-1 receptor kinase inhibitors as lead anti-neoplastic and anti-papilloma agents

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Abstract

The insulin-like growth factor-1 receptor (IGF-1 receptor) is a receptor tyrosine kinase, highly homologous to the insulin receptor. In contrast to the insulin receptor, which is mostly involved in metabolic pathways, the IGF-1 system plays a pivotal role in normal and neoplastic cell growth through anti-apoptotic, proliferative and metastatic pathways. Furthermore, IGF-1 receptor over-activation is found to correlate with a variety of tumors, such as breast cancer, prostate cancer, hematological malignancies, colorectal cancer and other proliferative diseases, such as psoriasis and papilloma. In addition, accumulating evidence implies that blockade of IGF-1 receptor activity causes reversal of tumor progression in cell lines as well as in animal tumor models. Because of the central role the IGF-1 receptor plays in oncogenic maintenance and metastatic processes, it is a highly appropriate target for anti-cancer agents. Here we report on a novel substrate-mimic family of IGF-1 receptor inhibitors. These compounds are tertiary aromatic amines, non-competitive with ATP and possess high affinity towards the IGF-1 receptor. The most potent compound, SBL02 inhibited the IGF-1 receptor with an IC_{50} of 170 nM in a cell-free kinase assay and was found to inhibit IGF-1 receptor auto-phosphorylation and substrate phosphorylation at the low micromolar range in cellular assays. SBL02 also blocks the formation of colonies in soft agar by cancer cells and inhibits the growth of keratinocytes and of HPV16 immortalized keratinocytes. This new family of non-ATP competitive, IGF-1 receptor inhibitors can serve as a lead for the development of anti-cancer, anti-psoriatic and anti-papilloma agents.

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Keywords: IGF-1 receptor; Kinase; Inhibitors

1. Introduction

The insulin-like growth factor-1 receptor (IGF-1 receptor) is a receptor tyrosine kinase, highly homologous to the insulin receptor (Hubbard, 1997). IGF-1 and its receptor play a pivotal role in normal and neoplastic cell growth through anti-apoptotic, and proliferative pathways (Baserga et al., 2003; Brodt et al., 2000; LeRoith and Roberts, 2003). IGF-1 signaling is enhanced in many tumors, such as breast cancer (Resnik et al., 1998), prostate cancer (DiGiovanni et al., 2000; O'Brien et al., 2001), colorectal cancer (Davies et al., 2006; Sandhu et al., 2002), thyroid cancer (Ciampolillo et al., 2005), tumors of the central nervous system (CNS) (Zumkeller and Westphal, 2001), lung cancer (Pavelic et al., 2005), multiple myeloma

(Mitsiades et al., 2004), chronic lymphocytic leukemia (Schillaci et al., 2005) and more. Malignant transformation is often associated with increased expression and/or constitutive activation of the IGF-1 receptor (Moschos and Mantzoros, 2002; Rubin and Baserga, 1995). For example, the highly metastatic murine lung carcinoma cells, H-59, express high IGF-1 receptor levels and this IGF-1 receptor over-expression was found to be critical for the ability of these cells to form metastases in the liver. H-59 cells expressing antisense RNA of the IGF-1 receptor were non-invasive, and failed to form metastases in the liver (Long et al., 1995). Expression of dominant negative IGF-1 receptor in Rat-1 fibroblasts inhibited tumorigenesis (Prager et al., 1994). Injection of dominant negative IGF-1 receptor utilizing a viral vector, showed significant growth suppression in established lung cancer xenografts (Lee et al., 2003). Blocking the IGF-1 receptor with specific antibody against the receptor inhibits the growth of human breast cancer cells (Arteaga and Osborne, 1989; Werner

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et al., 1993), breast cancer colony formation in soft agar (Hailey et al., 2002) and growth of Wilms tumor cells in culture and in nude mice (Gansler et al., 1989). Antisense RNA directed against the IGF-1 receptor mRNA lowered the expression of the IGF-1 receptor in human breast cancer cells and significantly decreased their growth rate (Neuenschwander et al., 1995), reversed and inhibited malignancy of cervical cancer cells (Nakamura et al., 2000), and rhabdomyosarcoma (RMS) cells (Shapiro et al., 1994). Since the IGF-1 receptor plays an important role in oncogenic maintenance and metastatic processes, it has been identified as a significant therapeutic target (Baserga, 2005; Hofmann and Garcia-Echeverria, 2005; O'Farrell et al., 2003; Pollak et al., 2004). A number of IGF-1 receptor kinase inhibitors have been reported, one of which shows anti-tumor activity in vivo (Mitsiades et al., 2004), but did not make it yet to clinical development.

Our group has argued that it may be beneficial to develop tyrosine kinase inhibitors that are not competitive with ATP (Levitzki, 2000a; Levitzki and Mishani, 2006; Litman et al., in press; Livnah et al., 2004). We have previously reported substrate competitive inhibitors of the IGF-1 receptor. Of these inhibitors, AG 538 was the most potent and some of the benzoxazolone catechol-mimics were quite potent (Blum et al., 2000, 2003). In view of the instability of AG 538 and the benzoxazolone inhibitors (unpublished) we searched for other scaffolds to generate substrate-mimics. In this study we report on a new family of high affinity inhibitors of the IGF-1 receptor that do not target the ATP site. These tertiary amine inhibitors show up to 5-fold selectivity vis-à-vis the insulin receptor and improved efficacy as inhibitors of tumor cells, in which the IGF-1 receptor is reported to play a major oncogenic role. The crystal structure of the IGF-1 receptor in its tris-phosphorylated, activated form, in complex with a peptide substrate and an ATP analog (Favelyukis et al., 2001), enabled us to dock drug candidates in the substrate-binding domain of the receptor, and helped us in the design of substrate-mimics, some of which are described in this article.

2. Materials and methods

Chemicals for organic synthesis, protease inhibitors, phosphatase inhibitors, immobilized lectin beads, *N*-acetyl-D-glucosamine, bovine serum albumin (BSA), poly (Glu, Tyr) 4:1 (pGT), 2, 2'-azido-bis 3-ethylbenzimidazole-6-sulfonic acid (ABTS), and IGF-1, di-phosphorylated mitogen activated protein kinase (MAPK) (phospho Erk), mouse monoclonal anti-phosphotyrosine clone PT-66 and peroxidase conjugate antibodies were all from Sigma. Anti-phospho-insulin receptor substrate-1 (IRS-1) antibody was from Oncogene, anti-IRS-1 from Upstate Biotechnologies. Anti-Protein Kinase B 1/2(PKB1/2), anti-Erk2 (Extracellular signal regulated kinase 2), anti-IGF-1 receptor β antibodies were from Santa Cruz Biotechnology, and anti-phospho-PKB (Thr 308) antibody from Cell Signaling Technology. Medium from a hybridoma producing anti-phosphotyrosine, 4G10, was used for immunoblotting. Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum were from Biological Industries Bet-Haemek Ltd., Israel. Dimethylsulfoxide (DMSO) was from BDH. Protein determination paper was from Whatman

Inc. USA. The REGRESSION program was from Blackwell Scientific Software, Osney Mead, Oxford UK. The Merck Hitachi HPLC included an L 6200 pump, a D 6000A interphase, L-4250 UV detector and AS-4000 autosampler. Integration employed the HSM HPLC System Manager, Merck KGaA, Darmstadt and Hitachi Instruments, Inc., San Jose. Reversed-phase preparative HPLC was performed with a C-18 column (218TPL022 Vydac). Mass spectrometry was performed using an LCQ^{DUO}, from ThermoQuest of Finnigan, and NMR was on a Bruker AMX 300.

All solvents for HPLC analysis and purification were from J. T. Baker, BDH or Bio-Lab Ltd. (Israel). Reagents for chemical synthesis were from Frutarom.

2.1. Organic synthesis

SBL01: 0.5 M (138 mg) 3,4 dihydroxybenzaldehyde in methanol (MeOH) mixed with 15 μ l acetic acid glacial (AcOH) was added drop-wise to 0.5 M (137 mg) 3,4-methylenedioxy aniline in MeOH and 200 mg of molecular sieve. These were stirred for 30 min, under nitrogen. 0.5 M (63 mg) NaCNBH₃ was added to the reaction mixture and stirred for an hour, under nitrogen. The reaction mixture was then filtered through 6.2 g silica gel 70–230 with 400 ml of 10% MeOH/chloroform, and subsequently evaporated, followed by dissolving in a minimal volume of MeOH and was precipitated in 400 ml diethyl ether.

The product was purified by HPLC preparative RP18 column. SBL01 was eluted by 22% acetonitrile in water, containing 0.1% trifluoroacetic acid (TFA), lyophilized to give 9 mg of white powder. ¹H NMR (DMSO-*d*₆, 300 MHz): δ =6.81 (m, 2H, CH_{arom}), 6.64 (m, 3H, CH_{arom}), 6.45 (m, 1H, CH_{arom}), 5.96 (s, 2H, OCH₂O), 4.16 (s, 2H, CH₂N).

MS (ES⁺): m/z =260 (MH⁺).

SBL02: To 0.5 M (553 mg, 2eq.) 3,4-dihydroxybenzaldehyde in MeOH mixed with 100 μ l AcOH was added drop-wise 0.5 M (275 mg) 3,4-methylenedioxy aniline in MeOH. These were stirred for 30 min. 0.5 M (255 mg, 2eq.) NaCNBH₃ was added to the reaction mixture and stirred for 3 h. The reaction mixture was then evaporated.

The product was purified by HPLC preparative RP18 column. SBL02 was eluted by 24% acetonitrile in water (containing 0.1% TFA), lyophilized to give 3 mg of light yellow powder. ¹H NMR (acetone-*d*₆, 300 MHz): δ =6.76 (m, 4H, CH_{arom}), 6.62 (m, 3H, CH_{arom}), 6.50 (s, 1H, CH_{arom}), 6.29 (m, 1H, CH_{arom}), 5.84 (s, 2H, OCH₂O), 4.54 (s, 4H, CH₂N).

MS (ES⁺): m/z =382 (MH⁺).

SBL03 and SBL04: To 0.5 M (553 mg, 2eq.) 3,4-dihydroxybenzaldehyde in MeOH mixed with 100 μ l AcOH was added drop-wise 0.5 M (278 mg) 6-amino indazole in MeOH. These were stirred for 30 min. 0.5 M (274 mg, 2eq.) NaCNBH₃ was added to the reaction mixture and stirred for 3 h. The reaction mixture was then evaporated. The products were purified by sequential HPLC preparative RP18 column separation. SBL03 was eluted by 22% acetonitrile in water (containing 0.1% TFA) and then lyophilized to give light yellow powder. ¹H NMR (acetone-*d*₆, 300 MHz): δ =7.76 (s, 1H, CH=N), 7.44

(d, 1H, $J=8.7$ Hz, CH_{arom}), 6.9 (m, 1H, CH_{arom}), 6.73 (m, 3H, CH_{arom}), 4.24 (s, 2H, CH₂N).

MS (ES⁺): $m/z=256$ (MH⁺).

SBL04 was eluted by 31% acetonitrile in water (containing 0.1% TFA) and then lyophilized to give light yellow powder. ¹H NMR (acetone-*d*₆, 300 MHz): $\delta=7.83$ (s, 1H, CH=N), 6.68 (m, 8H, CH_{arom}), 4.27 (m, 2H, CH₂N), 4.14 (m, 2H, CH₂N).

MS (ES⁺): $m/z=378$ (MH⁺).

SBL05 and SBL06: To 0.5 M (553 mg, 2eq.) 3,4-dihydroxybenzaldehyde in MeOH mixed with 100 μ l AcOH was added drop-wise 0.5 M (288 mg) 2-aminonaphtalene in MeOH. These were stirred for 30 min. 0.5 M (255 mg, 2eq.) NaCNBH₃ was added to the reaction mixture and stirred for 3 h. The reaction mixture was then evaporated. The products were purified by HPLC preparative RP18 column separation. SBL05 was eluted by 24% acetonitrile in water (containing 0.1% TFA) and then lyophilized to give white powder. ¹H NMR (DMSO-*d*₆, 300 MHz): $\delta=7.63$ (m, 2H, CH_{arom}), 7.53 (d, 1H, $J=8.1$, CH_{arom}), 7.3 (m, 1H, CH_{arom}), 7.12 (m, 1H, CH_{arom}), 7.07 (dd, 1H, $J=8.8$, $J=2.2$, CH_{arom}), 6.79 (m, 2H, CH_{arom}), 6.67 (s, 1H, CH_{arom}), 4.3 (s, 2H, CH₂N).

MS (ES⁺): $m/z=266$ (MH⁺).

SBL06 was eluted by 95% acetonitrile in water (containing 0.1% TFA) and then lyophilized to give white powder. ¹H NMR (acetone-*d*₆, 300 MHz): $\delta=7.84$ (m, 1H, CH_{arom}), 7.68 (m, 2H, CH_{arom}), 7.34 (m, 1H, CH_{arom}), 7.13 (m, 2H, CH_{arom}), 6.69 (m, 7H, CH_{arom}), 4.38 (s, 2H, CH₂N), 4.25 (s, 2H, CH₂N).

MS (ES⁺): $m/z=388$ (MH⁺).

3,4-diacetoxybenzaldehyde: 3,4-dihydroxybenzaldehyde, (5.4 g, 36 mmol), acetic anhydride 300 ml, 3.17 mol) and pyridine (6 ml) were refluxed 3 h at 100 °C, and then stirred for 3 days. The reaction mixture was added to 0.5 L of DDW, stirred for another 2.5 h and then the product was extracted with DCM (5 \times 100 ml against 100 ml DDW). The organic fraction was evaporated to minimal volume, dried over MgSO₄, filtered and kept at 4 °C overnight without sedimentation. Chromatography was performed on silica 35–70, 1:20 ratio. The product was eluted with 1% MeOH in dichloromethane (DCM) to give 3.1 g white solid. 39% yield. ¹H NMR (DMSO-*d*₆, 300 MHz): $\delta=10.04$ (s, 1H, HC=O), 7.90 (d, 1H, $J=8.1$, CH_{arom}), 7.84 (s, 1H, CH_{arom}), 7.54 (d, 1H, $J=8.1$, CH_{arom}), 2.32 (d, 6H, $J=2.1$, CH₃C=O).

MS (ES⁺): $m/z=223$ (MH⁺).

SBL07: 0.52 M (465 mg, 2.1 mmol) 3,4-diacetoxybenzaldehyde in MeOH mixed with 30 μ l AcOH was added drop-wise to 0.5 M (276 mg, 2 mmol) 3,4-methylenedioxy aniline in MeOH. These were stirred for 20 min. Then an additional 14 ml of MeOH were added. 5 min later, 4.1 g (2 mmol) of polymer-bound CNBH₃ beads were added to the reaction mixture and stirred for 3 h. The reaction mixture was then diluted with 200 ml of MeOH and stirred for another hour. The beads were filtered and washed with MeOH and the reaction mixture was evaporated.

Chromatography was performed on neutral alumina, 1:50 ratio. SBL07 was eluted with 10% EtAc in chloroform to give 195 mg brown solid. 27% yield. ¹H NMR (DMSO-*d*₆, 300 MHz): $\delta=7.23$ (m, 3H, CH_{arom}), 6.63 (d, 1H, $J=2.2$ Hz, CH_{arom}), 6.27 (d, 1H, $J=8.3$ Hz, CH_{arom}), 5.97 (m, 1H, CH_{arom}), 5.81 (s, 2H, OCH₂O), 4.2 (d, 2H, $J=6.2$, CH₂N), 2.26 (d, 6H, $J=1.3$, CH₃C=O).

MS (ES⁺): $m/z=344$ (MH⁺).

2.1.1. Reductive alkylation in chemical library synthesis

Compounds were analyzed by MS. Compound purity was determined by analytical RP C-18 HPLC.

Mono-alkylated library: In order to prepare the mono-alkylated library, the starting materials were dissolved in methanol. 200 μ l, 0.5 M (0.1 mmol) of each of the aldehydes were mixed with 3 μ l (0.5%) acetic acid and then added to 200 μ l, 0.5 M (0.1 mmol) of each of the amines in a 1 ml 96-well plate. After 30 min, 200 μ l, 0.5 M (0.1 mmol) of sodium cyano-borohydride were added for another 3 h of mixing. The library compounds were then dried by argon flow and dissolved in 500 μ l DMSO to obtain 200 mM of each product (assuming 100% yield).

Di-alkylated library: This library was synthesized according to the procedure described for library A, with the following adjustments: 1) the amines were added drop-wise to the aldehydes–acetic acid mixtures, not the opposite. 2) Acetic acid concentration was 0.25% of total volume, not 0.5%. 3) Total reaction time, after the addition of sodium cyano-borohydride was 24 h.

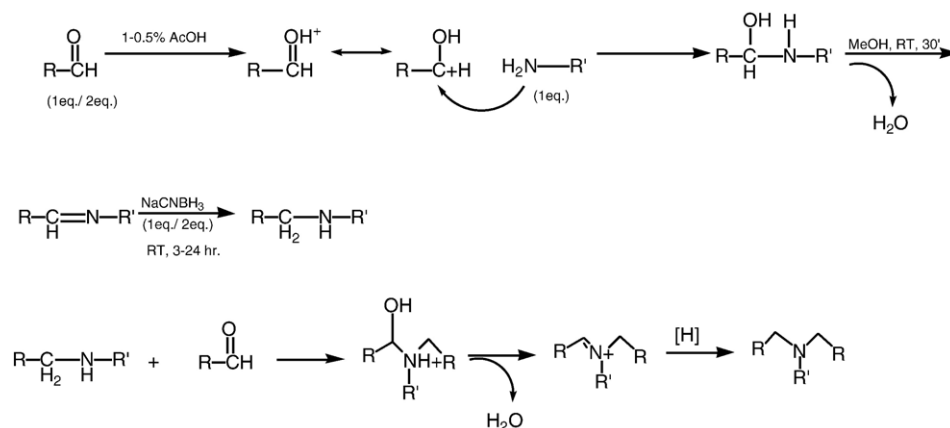
2.2. Purification of the IGF-1 receptor and insulin receptor

Purification of the IGF-1 receptor and the insulin receptor were based on the IGF-1 receptor purification method described earlier (Blum et al., 2003). Confluent R⁺ cells over-expressing the human IGF-1 receptor or WTIR cells over-expressing the human insulin receptor were lysed in the presence of 10% glycerol, 50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 5 μ M EGTA, 0.24 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin, 25 mM benzamidine, and 10 μ g/ml soybean trypsin inhibitor. The lysate was bound to immobilized lectin overnight at 4 °C and washed with 5 column volumes of HTN buffer (50 mM HEPES, 1% Triton X-100, and 150 mM NaCl). Additional washes were with 50 mM HEPES, 1% Triton X-100, 1 M NaCl and then with 10% glycerol/HTN. Semi-purified IGF-1 receptor or insulin receptor was eluted with 0.5 M *N*-acetyl-D-glucosamine in 10% glycerol/HTN, frozen, and kept at –70 °C. As reported earlier (Blum et al., 2003), in each of the preparations the only protein tyrosine kinase activity is IGF-1 receptor or insulin receptor kinase.

2.3. Inhibition of IGF-1 receptor catalyzed substrate phosphorylation

The inhibitory activities of new compounds were measured as described previously (Blum et al., 2003). Briefly, the general protein tyrosine kinase substrate Poly Glu₄Tyr (pGT) was coated onto a 96-well ELISA plate. Human IGF-1 receptor, human insulin receptor or GST-Src (human), were incubated, with 20 μ M ATP, 10 mM MgCl₂, 5 mM MnAc₂, with or without inhibitors, at various concentrations, then washed and blocked. For each kinase, the time of incubation was according to our published protocols (Blum et al., 2003; Karni et al., 2003).

Phosphorylated tyrosines were detected using mouse monoclonal anti-phosphotyrosine peroxidase conjugate and a color reagent, ABTS. IC₅₀ values of inhibitors were calculated



Scheme 1. The generation of secondary and tertiary amines by reductive alkylation.

using the REGRESSION program. A more detailed description is in the Supplementary material.

2.4. Cells

NIH-3T3 mouse fibroblast cells over-expressing human wild type IGF-1 receptor at approximately 700,000 receptors per cell (clones NWTc43) or insulin receptor (clone WTIR) were a generous gift from Dr. D. LeRoith. Cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 500 mg/ml geneticin (G418).

R^+ cells were a generous gift from Dr. R. Baserga. R^- cells are mouse embryo fibroblasts, which are 3T3-like cells devoid of IGF-1 receptor. R^+ cells were generated by stable transfection of IGF-1 receptor to R^- cells, and these cells express approximately 1×10^6 copies of IGF-1 receptor (D'Ambrosio

et al., 1997). Cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 50 $\mu\text{g/ml}$ G418, and 50 $\mu\text{g/ml}$ hygromycin B in a humidified atmosphere of 94% air and 6% CO_2 at 37 $^\circ\text{C}$.

Primary keratinocytes and immortalized keratinocytes (HF1) cells were maintained in Keratinocyte Growth Medium (KGM): DMEM, 25% Nutrient mixture F-12 (HAM), 10% fetal bovine serum (FBS), 5 $\mu\text{g/ml}$ insulin, 0.4 $\mu\text{g/ml}$ hydrocortisone, 10^{-10} M cholera toxin, 10 ng/ml EGF, 1.8×10^{-4} M adenine, 5 $\mu\text{g/ml}$ transferrin, 2×10^{-9} M T3, 100,000 U/L penicillin, 100 $\mu\text{g/L}$ streptomycin, 0.1 mg/ml amphotericin. Primary keratinocytes were cultured from small biopsy specimens, and were kindly provided by Dr. H. BenBassat. The HF1 cell line was derived from human keratinocytes transfected with the genome of the human Papilloma virus, HPV16 (Mitrani-Rosenbaum and Tsvieli, 1992). Early passage

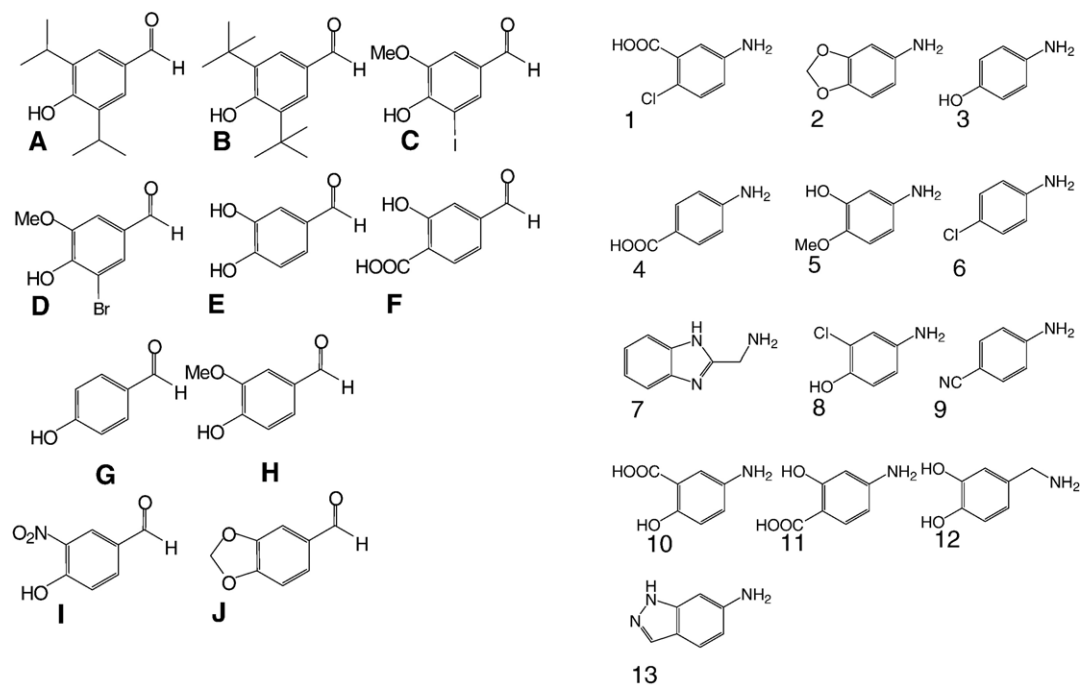


Fig. 1. Aldehydes and amines — the building blocks of the libraries. Aldehydes (A–J), shown in the left panel, amines (1–13) in the right panel. The first library comprised aldehydes A–J combined with amines 1–12. A second, optimized library comprised aldehydes E, F and J combined with amines 1–13.

(passage 20), which represents cells that underwent about 100 doublings, after transfection was used.

2.5. Inhibition of IGF-1 receptor auto-phosphorylation and downstream signaling in intact cells

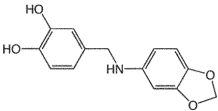
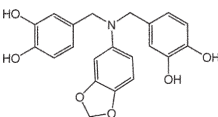
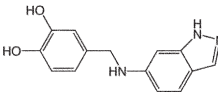
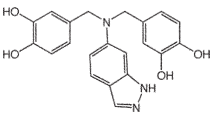
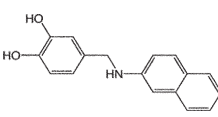
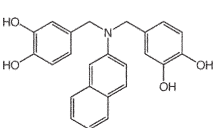
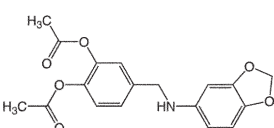
Tyrosine auto-phosphorylation of IGF-1 receptor and the IGF-1 receptor signaling was assayed similarly as described previously (Blum et al., 2003). Briefly, sub-confluent NWTc43 cells were incubated at 37 °C overnight with inhibitor at various concentrations and then starved in the presence of the inhibitors, for another hour at 37 °C. Cells were then stimulated with IGF-1 for 5 min. After IGF-1 treatment, cells were washed and lysed. Equal amounts of protein per lane were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylated proteins were immunoblotted with monoclonal anti phosphor-tyrosine, anti-phospho-IRS-1, anti-phospho-PKB (Thr 308) and anti-phospho-Erk antibodies, and detected with horseradish peroxidase-conjugated secondary antibody using the ECL system. Blots were then stripped of antibodies, washed and blocked. Blots that were probed with anti-phospho-tyrosine

were re-probed with anti-IGF-1 receptor. Other blots were re-probed with antibodies against the corresponding non-phosphorylated protein anti-PKB1/2 or anti-Erk2 antibodies. Blots with anti-phospho-IRS-1 were re-probed with anti-PKB1/2. Band intensities were quantified using the NIH image program. The amount of phosphorylated protein was divided by the amount of (phosphorylated plus non-phosphorylated) protein in each lane. This value was normalized to the maximal activity (designated as 100%) detected in the samples treated with IGF-1 without inhibitor. A more detailed description is presented in the Supplementary material.

2.6. Anchorage independent cell growth assay

Colony formation in soft agar was performed as described previously (Horowitz and King, 2000). The cell lines that were utilized were ones reported to depend on IGF-1 receptor: LNCaP, MCF-7 (Hailey et al., 2002; Maloney et al., 2003) and PC-3 (Maloney et al., 2003). The inhibitors were added at various concentrations to the soft agar plate, once only, in the growth medium, 24 h after plating. 12 days after plating,

Table 1
Inhibition of pGT phosphorylation, by pure inhibitors, in μM

| Name | Structure | IC ₅₀ of IGF-1 receptor | IC ₅₀ of insulin receptor | IC ₅₀ of Src |
|------------------|---|------------------------------------|--------------------------------------|-------------------------|
| SBL01 (E2-mono) |  | 1.69 ± 0.6 (4) | 2.08 ± 0.68 (4) | 1.86 |
| SBL02 (E2-di) |  | 0.17 ± 0.09 (4) | 0.61 ± 0.42 (4) | 0.83 |
| SBL03 (E13-mono) |  | 1.5 ± 0.75 (6) | 2.25 ± 0.45 (2) | ND |
| SBL04 (E13-di) |  | 0.18 ± 0.19 (4) | 0.38 ± 0.17 (2) | ND |
| SBL05 |  | 4.5 ± 3.7 (6) | 5.15 ± 2.85 (2) | ND |
| SBL06 |  | 2.45 ± 1.15 (2) | 1.6 ± 0.2 (2) | ND |
| SBL07 |  | 1.3 ± 0.3 (2) | 9.9 (1) | ND |

The inhibitors presented in the table were synthesized, purified and analyzed as described in the Materials and methods section. All inhibitors were over 95% pure according to HPLC analysis. IC₅₀ values were determined using ELISA for pGT phosphorylation, as described in the Materials and methods section. Figures in parentheses represent numbers of repetitions.

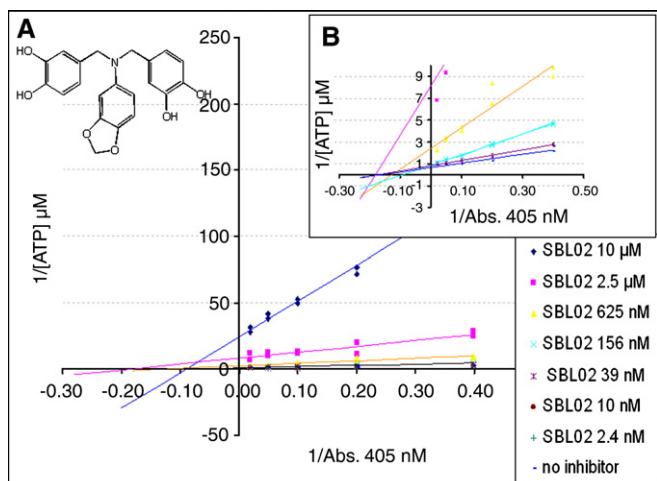


Fig. 2. SBL02 is non-competitive with ATP. The substrate pGT was phosphorylated by IGF-1 receptor as described in the Materials and methods section. IGF-1 receptor was assayed at concentrations of ATP ranging from 2.5 to 50 μM and SBL02 ranging from 0 to 10 μM (shown as different styled curves). All points represent 5 duplicates at each concentration, where all experimental points were used for analysis. The data was analyzed by the Lineweaver–Burk plot using the Excel program. A. All of the experimental data is shown. B. Insert: The higher the inhibitor concentration, the lower the enzyme activity is, leading to decreased accuracy of assay. Insert shows high resolution of the more accurate lower inhibitor concentrations, and shows convergence of curves at the X axis, between -0.1 and -0.2 mM^{-1} ATP.

cells were stained with MTT, photographed at 100 times magnification, and counted. The assays were performed in triplicate.

2.7. Anchorage dependent growth inhibition of keratinocytes

For growth curves, primary keratinocytes (7000 cells/well) and HF1 cells (8000 cells/well), were plated on 96-well plates in the appropriate media supplemented with 10% FBS, in a humidified atmosphere of 94% air and 6% CO_2 at 37 $^\circ\text{C}$. One or two days after seeding, the growth medium was replaced with SBL01, SBL02 or AG 538, as positive control, at various concentrations in growth medium. The final concentration of 0.1% DMSO was kept constant in all samples. Triplicate samples were utilized for each concentration. Cell growth was determined by the micro culture methylene blue assay as follows. Cells were fixed in glutaraldehyde, 0.05% final concentration, for 10 min at room temperature. After washing, the micro plates were stained with 1% methylene blue in 0.1 M borate buffer, pH 8.5, for 60 min at room temperature. The plates were then washed extensively and rigorously (8 times) to remove excess dye and dried. The dye taken up by cells was eluted in 0.1 M HCl for 60 min at 37 $^\circ\text{C}$, and absorbance monitored at 620 nm.

2.8. Docking of SBL02 to IGF-1 receptor active kinase

The pocket creator program, created by David Marcus of the Hebrew university of Jerusalem, was used to crop an 8A pocket around AMP-PCP of the IGF-1 receptor active kinase active site. SBL02 was built, minimized and docked to the active pocket of IGF-1 receptor kinase with the ArgusLab 4.0.1 program of Mark Thompson and Planaria Software LLC. Images were presented using the PyMOL program of DeLano Scientific LLC.

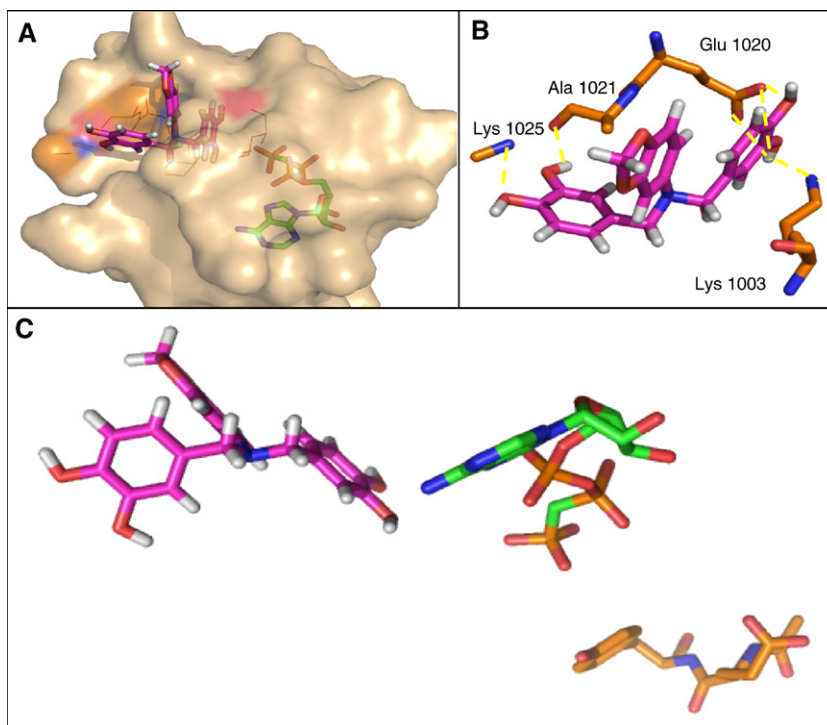


Fig. 3. SBL02 docked in the substrate site of the IGF-1 receptor kinase domain. SBL02 is shown in pink. AMP-PCP is shown in green. Docking was performed using ArgusLab 4.0. The figure was prepared using PyMOL. A. A complete view of the IGF-1 receptor kinase domain (represented by surface coloring), SBL02 and AMP-PCP. Hydrogen bonding residues are represented by lines. B. SBL02 with Hydrogen bonding residues of IGF-1 receptor kinase. Hydrogen bonding residues are shown in orange, Hydrogen bonds are represented by dotted yellow lines. C. SBL02 with AMP-PCP and a peptide of IRS-1. IRS-1 peptide is shown in orange.

3. Results

In order to improve lead compounds through comprehensive structure activity relationship (SAR) analysis, we utilized successive combinatorial chemical libraries. This approach of multi-parallel synthesis of small molecules has the advantage of generating a large number of products in parallel, using relatively simple straight-forward synthetic protocols. We generated mono and di-alkylated amines according to [Scheme 1](#). We optimized the reaction to 60–75% yield of the main product.

We utilized 10 different aromatic aldehydes combined with 13 different anilines and benzyl amines ([Fig. 1](#)) to generate mono-alkylated amines and di-alkylated amines, optimizing condensation/reduction conditions to generate both families ([Scheme 1](#)).

The crude compounds of the library as well as the starting materials were all tested for their potency to inhibit the IGF-1 receptor dependent phosphorylation of pGT at concentrations of 100 μ M, 33 μ M and 11 μ M inhibitor, using a cell free assay with purified human IGF-1 receptor, insulin receptor ([Blum et al., 2000](#)) and GST-Src ([Karni et al., 2003](#)).

After purification of the active compounds by semi-preparative HPLC and analysis, we found that the most active compounds were actually the di-alkylated byproducts of the first reaction in [Scheme 1](#). Di-alkylation is a known side reaction of the reductive alkylation reaction: the secondary amine product of the initial reaction attacks another aldehyde to produce a tertiary amine ([Scheme 1](#), bottom). These results indicated to us that an additional ring is most probably beneficial to generate higher affinity inhibitors of activated IGF-1 receptor. We therefore

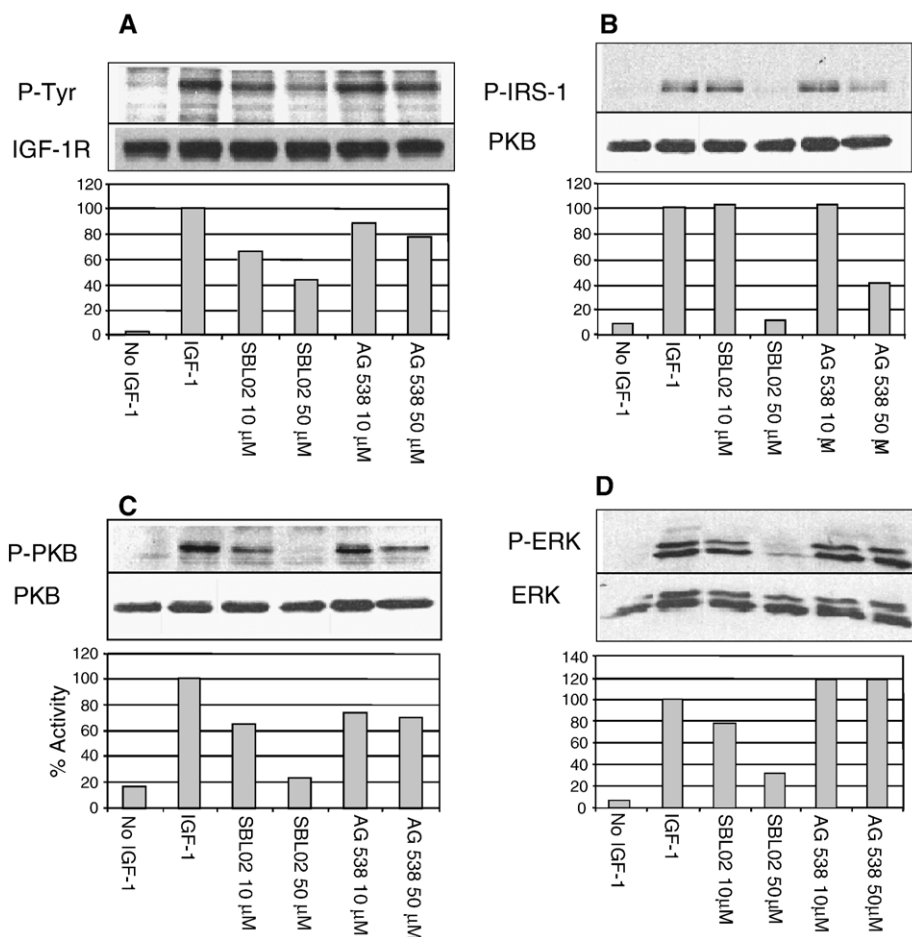


Fig. 4. Inhibition of IGF-1 receptor signaling in intact cells by SBL02. NIH-3T3 cells over-expressing the IGF-1 receptor were incubated for 16 h with the inhibitors SBL02 or the positive control, AG 538. Incubation was followed by one-hour starvation in the presence of inhibitors. 50 ng/ml IGF-1 was added for 5 min. Cells were then lysed and equal amounts of protein separated by SDS-PAGE and transferred to a nitrocellulose membrane. **A.** Inhibition of IGF-1 receptor auto-phosphorylation. Western blot using anti-phospho-tyrosine 4G10 antibody. Re-probing of stripped blot with α -IGF-1 receptor antibody. The phosphorylation level of each sample was normalized to the IGF-1 receptor level. These values are shown as percentages of the level of auto-phosphorylation in the absence of inhibitor (designated 100%). **B.** Inhibition of IRS-1 phosphorylation. Western blot using anti-phosphotyrosine-IRS-1 antibody. Re-probing of stripped blot with anti-IRS-1 antibody. The percent of maximum phosphorylation for each sample was defined in the same manner described in A, except the intensities of the bands obtained with anti-phospho-tyrosin IRS-1 were normalized to the intensities of the anti-PKB1/2 bands. **C.** Inhibition of PKB phosphorylation. Western blot using anti-phospho-PKB (Thr-308) antibody. Re-probing of stripped blot with anti-PKB1/2 antibody. The percent of maximum phosphorylation for each sample was defined in the same manner described in A, with normalization of phospho-PKB to total PKB1/2. **D.** Inhibition of Erk2 phosphorylation. Western blot using anti-di-phospho-Erk1 and 2 antibody. Re-probing of stripped blot with anti-Erk2 antibody. The percent of maximum phosphorylation for each sample was defined in the same manner described in A, with normalization of phospho-Erk to total Erk2. The experiment was repeated three times, and the figure shows a representative one blot.

optimized the synthetic procedure to favor the synthesis of the di-alkylated product, namely the tertiary amine (Scheme 1, bottom). The structures and IC_{50} for inhibition of the IGF-1 receptor of the crude compounds, in the *in vitro* pGT phosphorylation assay, is shown in supplementary data (supplementary data Table S1). In order to establish whether the catechol di-alkylated products were superior to the catechol mono-alkylated products, we focused on the most active inhibitors, out of the two libraries of the mono-alkylated and the di-alkylated products. As shown in Table 1 the three-di-alkylated products were more potent than the mono-alkylated ones. For example, SBL04 and SBL02 exhibited one order of magnitude higher potency than their mono-alkylated homologs, where the naphthalene moiety was less potent than the methylenedioxy or benzindazole moieties. SBL07, which was synthesized as protected SBL01 in order to improve SBL02 synthetic yield, was also rather active (Table 1).

3.1. Selectivity of the inhibitors

The inhibitors were also tested for potency against kinases with profound homology to the IGF-1 receptor kinase. The inhibitors were most active against the IGF-1 receptor, and were, interestingly, several folds less potent against the insulin receptor and pp60^{Src} (Table 1).

3.2. Mode of SBL02 inhibition

We characterized further the most active inhibitor, SBL02. Fig. 2 shows that SBL02 is non-competitive with ATP, as revealed by the Lineweaver–Burk plot. Docking studies revealed that SBL02 could easily be accommodated at the substrate-binding domain of the active form of the IGF-1 receptor, even in the presence of AMP-PCP (Fig. 3). Docking studies also showed that SBL02 did not dock at the ATP binding site. As expected, SBL02 also docked quite well to the substrate site of the activated insulin receptor (data not shown).

3.3. The inhibition of the IGF-1 receptor and its signaling in intact cells

SBL02 inhibits IGF-1 receptor mediated pathways in intact cells, with greater potency than the positive control AG 538. SBL02 inhibits IGF-1 induced receptor auto-phosphorylation, IRS1-phosphorylation and the activation of its downstream target, PKB, as measured by IGF-1 dependent phosphorylation of PKB (Fig. 4).

3.4. SBL02 inhibits colony formation in soft agar

In a soft agar assay, SBL02 inhibited with very good potency colony formation by three cell lines, in which IGF-1 receptor had been reported previously to play a significant role in the oncogenic phenotype (Djavan et al., 2001; Hailey et al., 2002; Haluska et al., 2006; Kawada et al., 2006; Kimura et al., 1996; Maloney et al., 2003; McVittie et al., 2006; Modha et al., 2003; Ngo et al., 2003; Papatsoris et al., 2005; Sachdev et al., 2003) (Fig. 5). This result is especially impressive, as the inhibitor was applied only once.

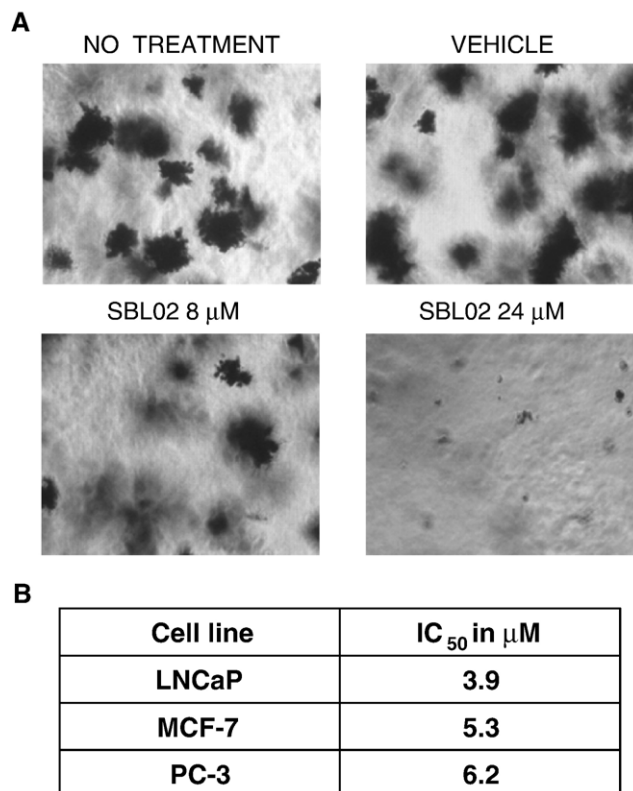


Fig. 5. Inhibition of colony formation in soft agar by SBL02. A suspension of separated LNCaP, PC-3 and MCF-7 cells was plated in growth medium containing 0.3% agar on top of a layer of growth medium containing 1% agar. One day later, growth medium supplemented with inhibitors at the appropriate concentrations was added. 7 to 12 days after plating (depending on the cell line), colonies were stained with MTT, photographed and counted. The IC_{50} value of SBL02 in each cell line was calculated. A. Representative examples of colony formation inhibition in LNCaP after 12 days. B. IC_{50} values for the three indicated cell lines. These experiments were repeated 3 to 4 times and the IC_{50} values represent a characteristic experiment where the values did not differ from each other by more than 20%. The values depicted are the ones for the experiment shown.

3.5. Inhibition of the growth of keratinocytes and of HPV16 immortalized keratinocytes

We have shown earlier that tyrphostins that inhibit the epidermal growth factor receptor (EGFR) and the activation of the cell cycle are potent inhibitors of psoriatic keratinocytes (Powell et al., 1999) and of HPV16 immortalized keratinocytes (Ben-Bassat et al., 1997). In view of the role of IGF-1 in the growth, survival and movement of keratinocytes (Hyde et al., 2004; Rudman et al., 1997; Tavakkol et al., 1992) we explored the activity of the new inhibitors on the growth of keratinocytes and of HPV16 immortalized keratinocytes (Fig. 6). It can be seen that also in this case the di-alkylated inhibitor SBL02 is more potent than its parent mono-alkylated derivative SLB01, as a growth inhibitor of HPV16 immortalized keratinocytes.

4. Discussion

In this study we have discovered a new family of substrate-mimetic inhibitors of the IGF-1 receptor. These compounds

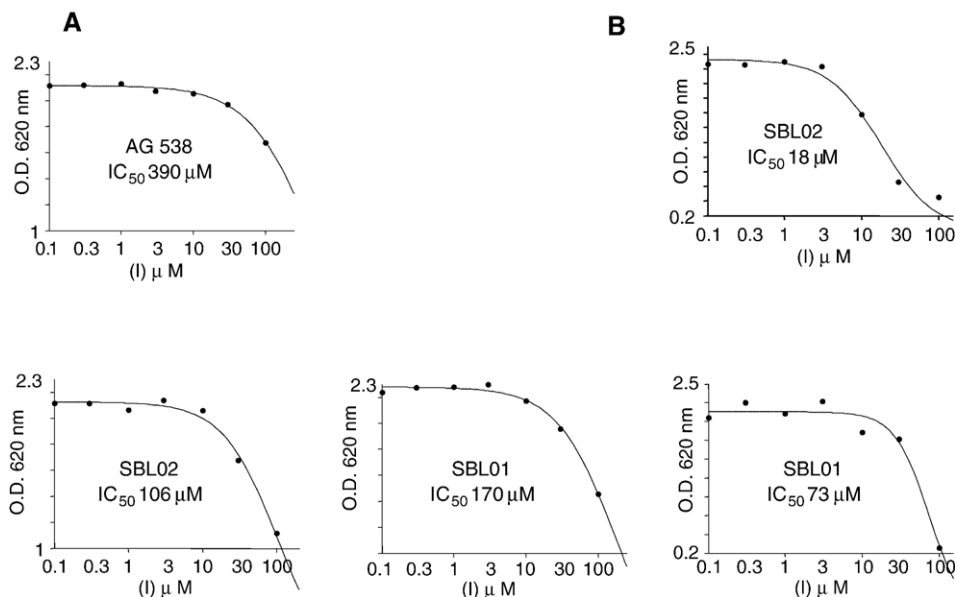


Fig. 6. Inhibition of keratinocytes growth by SBL01, SBL02. Cells were grown in 10% FBS in the presence or absence of the indicated inhibitors at the indicated concentrations, for 48–72 h. Cell density was determined by the methylene blue method. IC₅₀ values were calculated using the REGRESSION program, and IC₅₀ curves and values are shown above. A. Growth inhibition of immortalized keratinocytes (HF1). Immortalized keratinocytes were grown for 48 h. B. Growth inhibition of primary keratinocytes. Primary keratinocytes were grown for 72 h. Results are the average of three independent experiments.

(supplementary data Table S1, Table 1) are tertiary aromatic amines with relatively high affinity and are ATP non-competitive inhibitors of the IGF-1 receptor (Fig. 2). Docking studies strongly suggest that the compounds depicted in Table 1 bind to the substrate binding site of the activated IGF-1 receptor (Fig. 3). These agents are cell permeable, able to inhibit IGF-1 induced IGF-1 receptor signaling (Fig. 4) and potently inhibit, even after a single application, the growth in soft agar of a number of tumor cell lines (Fig. 5), for which the pro-growth/anti-apoptotic role of IGF-1 is established (Djavan et al., 2001; Hailey et al., 2002; Haluska et al., 2006; Kawada et al., 2006; Kimura et al., 1996; Maloney et al., 2003; McVittie et al., 2006; Modha et al., 2003; Ngo et al., 2003; Papatsoris et al., 2005; Sachdev et al., 2003) (Fig. 5). These compounds also exhibit reasonable potency in inhibiting the growth of keratinocytes and very good potency in inhibiting HPV16 immortalized keratinocytes (Fig. 6). The dialkylated amine SBL02 is superior to its mono-alkylated homolog SBL01 as an inhibitor of keratinocytes and HPV16 immortalized keratinocytes (Fig. 6), a similar relationship observed for the inhibition of IGF-1 receptor (Table 1). Although the selectivity of this class of inhibitors towards the IGF-1 receptor is satisfactory, both in the cell free assay (Table 1) and in the intact cell phosphorylation assays (Fig. 4) it inhibits insulin receptor and Src at concentrations only a few fold lower in the cell free assays. In fact its ability to inhibit the insulin receptor probably suggests that this class of compounds may also target mixed dimers between insulin receptor and IGF-1 receptor. This is a favorable feature, since such mixed dimers are considered to be important for the oncogenic activity of IGF-1 receptor (Kalli et al., 2002; Pandini et al., 2002). Although we chose for the anchorage independent growth inhibition cell lines that had been reported to depend on IGF-1 receptor for growth (Djavan et al., 2001; Hailey et al., 2002; Haluska et al., 2006; Kawada et al., 2006; Kimura et al., 1996;

Maloney et al., 2003; McVittie et al., 2006; Modha et al., 2003; Ngo et al., 2003; Papatsoris et al., 2005; Sachdev et al., 2003) one cannot rule out the possibility that this class of compounds also hits yet unknown pro-growth/anti-apoptotic modules. Similarly, the potency of SBL02 to inhibit the growth of HPV16 infected human keratinocytes may not be due to the exclusive inhibition of the IGF-1 receptor.

This class of IGF-1 receptor kinase directed typhostins (Table 1) therefore qualifies for development as anti-cancer agents, anti-psoriatic and anti-papilloma agents. There is an unmet need for IGF-1 receptor kinase inhibitors, since enhanced activity of this receptor is the hallmark of a significant number of solid tumors as well as hematological malignancies (see Introduction). It is generally believed that the role of the IGF-1 receptor in tumors is largely as a major contributor to the tumor anti-apoptotic shield. Therefore, inhibitors of the IGF-1 signaling are likely to be used in combination with pro-apoptotic anti-cancer agents. We advocate the development of inhibitors which are non-competitive with ATP, since they are apparently less toxic and do not need to compete with high intracellular ATP concentration (Levitcki, 2000a,b, 2003a,b,c) which leads to accelerated wash out from the kinase domain, as directly demonstrated for quinazolines in EGFR over-expressing tumors (Bonasera et al., 2001). A potent pyrrolo [2,3-*d*]pyrimidine derivative small molecular weight IGF-1 receptor kinase inhibitor (Mitsiades et al., 2004) was reported recently, but its clinical development was halted because of toxicity issues. It is interesting to note that it has been argued time and again that substrate competitive inhibitors are difficult to make and are not likely to succeed (Garber, 2006). This has now been refuted by the development of highly potent Bcr-Abl substrate competitive inhibitor (Reddy and Grove, 1998) and a PKB inhibitor (Litman et al., in press; Livnah et al., 2004). The current study supports the claim that one can achieve a high affinity tyrosine

kinase inhibitor that does not bind to the currently favorite ATP binding site.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2007.01.052.

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