DEVELOPMENT OF NEW IGF-1 RECEPTOR KINASE INHIBITORS USING CATECHOL MIMICS

By

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ABSTRACT

Since IGF-1 and its receptor play a pivotal role in many cancers it is an attractive target for the design of inhibitors. Here we present a new family of bioisostere inhibitors, based on the structure of AG 538. AG 538 is a substrate competitive inhibitor of the IGF-1 receptor, with an $IC_{50} = 61 \text{ nM}$ in a cell-free kinase assay [Blum et al. 2000]. AG 538 is a low molecular weight compound containing two catechol rings, which are sensitive to oxidation in cells. We have therefore prepared and examined catechol bioisosteres of AG 538. These AG 538 bioisosteres possess similar biological properties to AG 538: they inhibit IGF-1R by a substrate competitive mechanism and are non-competitive vis-à-vis ATP. They inhibit IGF-1R kinase activity in the sub-micromolar concentration range in cell-free assays. IGF-1 induced IGF-1R autophosphorylation; IRS-1 phosphorylation and PKB activation are inhibited at low micromolar concentration range when applied to intact cells. These inhibitors also block the formation of colonies in soft agar by prostate and breast cancer cells. The ability to replace catechol groups with a moiety that is more stable in cells may aid in developing non-catechol containing substrate competitive inhibitors targeted towards IGF-1R and possibly against other protein tyrosine-kinases (PTKs).

INTRODUCTION

The insulin-like growth factor-1 receptor (IGF-1R) and the insulin receptor (IR) are closely related members of the receptor tyrosine kinase superfamily. Both receptors are $_{2}$ heterodimers where the subunit contains the cytoplasmic kinase domain, which exhibits 84% identity between the two receptors. Upon ligand binding to the extracellular subunits, the subunits undergo trans autophosphorylation, which leads to receptor activation and phosphorylation of downstream substrates (1).

The IGF-1R is essential for normal growth and development, differentiation and mediates signals for the suppression of apoptosis, enhancement of mitogenesis and anchorage-independent growth (2). The main mechanism by which IGF-1R protects cells from apoptosis is via the activation of phosphatidylinositol-3 kinase (PI3K)/ Akt/ PKB pathway. An additional pathway was found in which IGF-1R protects cells from apoptosis by interacting with 14.3.3 proteins, causing the translocation of c-Raf to the mitochondria (3). In addition to these pathways, the IGF-1R promotes mitogenesis by activating the Erk/ MAPK pathway (4).

Malignant transformation is often associated with increased expression and/or constitutive activation of the IGF-1R (5). For example, the highly metastatic H-59 Lewis Lung carcinoma cells, express high IGF-1R levels, and this IGF-1R over expression was found to be critical for the ability of these cells to form metastases in mice livers. H-59 cells expressing antisense RNA of the IGF-1R became non-invasive, and failed to form metastases in the mice livers (6). Also an IGF-1R blocking antibody inhibits the growth of human breast cancer cells (7,8) and the formation of colonies in soft agar of these cells (9). These antibodies also inhibit the growth of Wilms tumor cells in culture and in nude mice (10).

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Expression of dominant negative IGF-1R or expression of antisense RNA directed against the IGF-1R mRNA in various cancerous cell lines caused inhibition of the transformed phenotype as detected by the inhibition of colony formation in soft agar or the inhibition of tumor formation in nude mice. The cell lines inhibited include: human cervical cancer (11), human prostate cancer (12), human gliomas cells (13), Rat-1 fibroblasts (14) and human rhabdomyosarcoma (RMS) cells (15). Enhanced IGF-1R signaling has also been implicated in the development and progression of prostate cancer (16).

For all these reasons, we have been trying to generate IGF-1R kinase inhibitors as potential anti neoplastic agents (17,18). We previously reported on a substrate competitive inhibitor of the IGF-1R, AG 538. AG 538 inhibits the IGF-1R with an IC_{50} of 61 nM in a cell-free kinase assay, and IGF-1 receptor autophosphorylation, as well as the activation of the downstream targets PKB and Erk2 in intact cells (18).

The catechol moiety present in AG 538 is unstable due to its vulnerability to oxidation (19). We therefore sought to generate substrate competitive inhibitors in which the catechol moiety is replaced by bioisosteres (20). Here we report on the synthesis of a family of AG 538 analogs in which the catechol group was replaced with a benzoxazolone group on either side of the molecule. The benzoxazolone group can in principle function as a bioisostere of the catechol moiety, maintaining the bioactivity of the compound (21). The successful replacement of the catechol moieties in AG 538 with benzoxazolone groups are likely to be leads for the development of new class of IGF-1R kinase substrate competitive inhibitors.

EXPERIMENTAL PROCEDURES

Reagents and instruments

All chemicals used for chemical 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-ethylbenzithiazoline-6-sulfonic acid (ABTS), IGF-1, PDGF, methylene blue, 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide; 3-(4, 5-dimethyl-2-thiazolyl) -2,5- diphenyl- 2Htetrazolium bromide (MTT) and diphosphorylated MAP kinase antibodies (phospho-Erk) were all from Sigma USA. Anti-phospho-IRS-1 antibody was from Oncogene research products, Germany, anti-IRS-1 from Upstate biotechnology, USA. Anti-Akt1/2, anti-Erk2, anti-IGF-1R ß antibodies were from Santa Cruz Biotechnology, USA, and anti-phospho-Akt (Thr 308) antibody from Cell Signaling Technology, USA. Medium from a hybridoma producing anti-phosphotyrosine, 4G10, was used for immunoblotting. Rabbit polyclonal anti-phosphotyrosine serum from SUGEN Inc. USA, was used for cell free catalyzed substrate phosphorylation assays. Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum were from Biological Industries Bet-Haemek Ltd., Israel. DMSO was from BDH, UK. ATP [³²P] was purchased from Amersham Life Sciences, UK, 3MM paper for radioactive assay from Whatman Inc. USA. The REGRESSION program was from Blackwell Scientific Software, Osney Mead, Oxford UK. The Merck Hitachi HPLC included a pump L-6200 and UV detector L-4250. Integration employed Varian Star 4.0 Star chromatography software from Varian Associates Inc. Reversed-phase HPLC was performed with an analytic C-18 column, 218TP54 Vidak, semipreparative C-18 column, Lichospher 100 (100µM) 618503 Merck Hitachi, preparative C-18 column,

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218TPL022 Vidak. Mass spectrometry was performed using an LCQ^{DUO}, from ThermoQuest of Finnigan, and NMR was performed on a Bruker AMX 300. All solvents for HPLC use were from J. T. Baker, USA, reagents for chemical synthesis were from Frutarom Israel.

Cell culture

NIH-3T3 mouse fibroblast cells overexpressing wild type IGF-1R at approximately 700,000 receptors per cell (clones NWTc34 and NWTc43) or insulin receptor (clone WTIR) were a generous gift from Dr. D. LeRoith (22). Cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 500 μ g/ml geneticin (G418), in a humidified atmosphere of 94% air and 6% CO₂ at 37^oC. R⁺ and R⁻ cells were a generous gift from Dr. R. Baserga. R⁻ cells are mouse embryo fibroblasts, which are 3T3-like cells devoid of IGF-IR (23). R⁺ cells were generated by stable transfection of IGF-1R to R⁻ cells, these cells express approximately 1X10⁶ copies of IGF-1R (23). Cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin 50 μ g/ml G418 and 50 μ g/ml hygromycin B, in a humidified atmosphere of 94% air and 6% CO₂ at 37^oC.

MDA MB-468 is a breast cancer cell line, a generous gift from Prof. Axel Ullrich, from the Max Planck Institute (Martinsreid, Germany). These cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin in a humidified atmosphere of 94% air and 6% CO₂ at 37^oC.

PC-3, obtained from the ATCC, and LNCaP, obtained from CaPCURE Israel, both are prostate adenocarcinoma cell lines. MCF-7 is a mammary carcinoma cell line obtained from the ATCC. PC-3 cells were cultured in DMEM, LNCaP cells were cultured in RPMI 1640 with 5µg/ml insulin and 1 nM testosterone. MCF-7 cells were

cultured in RPMI 1640. Media of all cell lines were supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin in a humidified atmosphere of 94% air and 6% CO₂ at 37^oC.

Partial purification of the IGF-1R and IR

Purification of the IGF-1R and IR were performed based on the IR purification method described earlier (24) and (18). Confluent R⁺ cells overexpressing the IGF-1R or WTIR cells overexpressing the IR 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 AEBSF, 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-1R or IR was eluted with 0.5 M N-Acetyl-D-glucosamine in 10% glycerol/HTN, frozen and kept at -70°C.

Inhibition of IGF-1R or IR catalyzed substrate phosphorylation

The general PTK substrate poly (Glu, Tyr) 4:1, (pGT), was coated onto a 96-well Maxisorp plates (Nunc) by adding 125 μ L 0.1 mg/ml PGT in PBS to each well. Plates were sealed and incubated for 16 h at 37°C, washed once with TBST (10 mM Tris/HCl pH 7.5, 50 mM NaCl and 0.1% Triton X-100), dried for 2-3 hours and stored at 4°C. Semipurified IGF-1R from R⁺ or IR from WTIR cells were prepared as described (18). The receptor was incubated (10 ng/well) in 20 μ M ATP, 10 mM MgCl₂, 5mM MnAc₂, and 20 mM Tris HCl pH 7.4, with or without inhibitors, for 20 minutes at 30°C. The plate was then washed with TBS with 0.2% tween-20 (TBST)

and blocked with 5% low-fat milk (1%). Rabbit polyclonal anti-phosphotyrosine serum (1:3000) was added to the plate, with incubation for 45 minutes at room temperature. The plate was washed repeatedly with TBST and then treated with anti-rabbit peroxidase conjugate antibody for 30 minutes. Detection was carried out with a color reagent, ABTS, in citrate-phosphate buffer, pH 4.0 with 0.004% H_2O_2 for 10 min and monitored at 405 nm, all at room temperature. IC₅₀ values of inhibitors were calculated using the REGRESSION program.

The assay was optimized with respect to the amount of IGF-1R or IR, reaction time and ATP concentration. The signal was linear for 30 minutes in the range of IGF-1R/IR protein concentrations up to 35 ng/well (18).

Inhibition of IGF-1R substrate phosphorylation by radioactive methods

200 ng/sample of semi-purified IGF-1R from R⁺ cells were added to a solution containing inhibitor and pGT at various concentrations. The reaction was initiated by the addition of reaction buffer (30 mM HEPES, 12 mM MgCl₂, 0.04 mM NaVO₃, 5 mM Mn (Ac)₂, 125 μ M ATP and 1.5 μ Ci/sample ATP [³²P], final concentration) at 30°C for 10 min. The reaction was stopped by the addition of EDTA pH=8, 0.1 M final concentration. Reaction samples were absorbed onto 3MM Whatman paper squares. The paper squares were then washed in 10% trichloroacetic acid (TCA), 1% sodium pyrophosphate at room temperature and dried in ethanol. Cerenkov radiation was measured by a 1600CA TRI-CARB Packard liquid scintillation counter. The data was analyzed by a Lineweaver–Burk plot using the Microsoft Excel program (18).

Inhibition of autophosphorylation of IGF-1R and IGF-1 signaling in intact cells

Tyrosine autophosphorylation of the -subunit of IGF-1R and the IGF-1R signaling was assayed as described (18). Briefly, sub-confluent NWTc43 or PC-3 or MDA MB-468 cells in 6-well polypropylene plates were incubated for 17 hours with inhibitor at various concentrations in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml, 0.1%DMSO and 0.1% ethanol (This final concentration of DMSO/ethanol was kept constant in all samples), in a humidified atmosphere of 94% air and 6% CO₂ at 37° C. Cells were then starved by incubation at 37° C for 1 hour in DMEM, containing inhibitors at the same concentration as before, in 0.1% DMSO and 0.1% ethanol. Cells were then stimulated with 50 ng/ml IGF-1 for 5 min. After IGF-1 treatment, cells were washed twice with ice-cold PBS and lysed by the addition of boiling sample buffer (10% glycerol, 50 mM Tris HCl pH 6.8, 3% sodium dodecyl sulfate and 5% -mercaptoethanol). Lysates were boiled for 15 minutes, and clarified by a 10 second 18000 g centrifugation at room temperature. Equal amounts of protein per lane were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Sartorius Germany). Phosphorylated proteins were immunoblotted with monoclonal anti phosphotyrosine, anti-phospho-IRS-1, anti phospho-Akt (Thr 308) and anti phospho-Erk antibodies. Detection was performed with horseradish peroxidase-conjugated secondary antibody using the ECL system. Blots were then stripped of antibodies by incubating in 2% SDS, 10 mM - mecaptoethanol, 62.5 mM Tris HCl pH 6.8 at 55 °C for 20 minutes, washed with TBS with 0.2% tween-20 (TBST) then blocked with TBST with 5% low fat milk and re-probed. Blots that had been probed with anti phosphotyrosine were re-probed with anti IGF-1Rß. Other blots were re-probed with antibodies against the corresponding non-phosphorylated protein anti IRS-1, anti Akt1/2 and anti Erk2 antibodies (e.g. the blots with anti phospho-IRS-

1 was re-probed with anti IRS-1). Band intensities were quantified using the NIH image program. To determine IC_{50} values of inhibition of PKB activation, the PKB levels in these samples divided the amount of phosphorylated PKB. This value was normalized to the maximal activity (designated as 100%) detected in the samples treated with IGF-1 without inhibitor. IC_{50} values were calculated using the REGRESSION program.

Activation of PKB phosphorylation by PDGF in intact cells

The activation of PKB by PDGF was performed as described above for activation by IGF-1, with the following changes. Sub-confluent NIH3T3 cells in 6-well polypropylene plates were incubated for 20 hours with inhibitor at various concentrations in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml, 0.1%DMSO (This final concentration of DMSO was kept constant in all samples), in a humidified atmosphere of 94% air and 6% CO₂ at 37°C. Cells were then starved by incubation at 37°C for 1 hour in DMEM, containing inhibitors at the same concentration as before, in 0.1% DMSO. Cells were then stimulated with 50 ng/ml PDGF for 5 min. After PDGF treatment, cells were washed twice with ice-cold PBS and lysed by the addition of boiling sample buffer. Lysates were boiled for 15 minutes, and clarified by short centrifugation at room temperature. 50 µg of protein per lane were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane.

Phosphorylated PKB was immunoblotted with anti phospho-Akt (Thr 308). Detection was performed with horseradish peroxidase-conjugated secondary antibody using an ECL system. Blots were then stripped of antibodies, washed with TBST then blocked with TBST with 5% milk and re-probed with anti Akt1/2 and detected as above.

Anchorage dependent growth inhibition

For growth curves, R^+ cells (450 cells/well), MDA MB-468 cells (2000 cells/well) and MCF-7 cells (2000 cells/well), were plated on 96-well plates in the appropriate media supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml, in a humidified atmosphere of 94% air and 6% CO₂ at 37°C. One day after seeding, cultures were treated with # 4 or # 10 or AG 538 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. Medium with and without inhibitors was replaced every day. Cell growth was determined by the micro culture methylene blue assay (25). Cells were fixed in glutaraldehyde, 0.05% final concentration, for 10 minutes at room temperature. After washing, the microplates 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°C, and absorbance monitored at 620 nm.

Anchorage independent cell growth assay

Colony formation in soft agar was performed mainly as described previously (26). A suspension of separated MDA MB-468, PC-3, LNCaP or MCF-7 cells was plated in agar at a density of 5000 cells/well in a 96-well plate in growth medium containing 0.3% agar (50µl/well), on top of a layer of growth medium containing 1% agar (100µl/well). One day later, 50 µl of growth medium supplemented with inhibitors at 4 times the final desired concentration was added on top of each cell line. The final concentration of 0.1% DMSO was kept constant in all samples. 7-12 days after

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

Chemistry

The aldehyde benzoxazolones were prepared from the acetal protected nitro hydroxy aldehydes. The nitro group was reduced and benzoxazolone formed with phenyl chloroformate instead of urea (Scheme 1) (27). In addition to the higher yields and less drastic conditions, this has the advantage of yielding the isomeric benzoxazolone aledhydes, **# 3** and **# 9**. Friedel-Crafts acetylation of benzoxazolone yields only the 6-acetyl isomer (28). The bromo acetyl benzoxazolone **# 13** was prepared by this route. The isomeric bromo acetyl **# 19** required the preparation of the 3-nitro 4-hydroxy acetophenone **# 16**, which led to the 5-acetyl benzoxazolone-isomer (Scheme 2). An attempt to obtain the bromo acetyl-benzoxazolone **# 13** with NBS or Br₂ failed. Only ring bromination on the benzoxazolone occurred. The bromo acetyl benzoxazolone **# 13** was prepared by Friedel-Crafts reaction of benzoxazolone with bromo acetyl bromide. We failed to obtain **# 14** by condensation of cyano-acetic acid to benzoxazolone with PPA.

The yields in the reactions of the bromo acetyl **# 13** and **# 19** with KCN were low or failed (11% and 0% respectively). We therefore tried to improve the yield with LiCN (29) and NaCN. The higher yields with LiCN (19% and 22% with **# 13** and **# 19**) do not justify the preparation of LiCN and the anhydrous conditions, and the commercial NaCN gave **# 20** at 29% yield, so we chose this latter route.

Tyrphostins were prepared by Knoevenagel condensation of the appropriate aldehydes and cyano ketones in the presence of alanine. By this condensation 11

tyrphostins (# 4, # 5, # 6, # 10, # 11, # 12, # 15, # 21, # 23, # 28 and # 29) were prepared (Scheme 1-5).

Workup means, adding water, extraction with CH₂Cl₂, and evaporation.

1. 3-hydroxy 4-nitro benzaldehyde (2.66 g, 16 mM), 1,3-dihydroxy 2,2-dimethyl propane (2.7 g, 26 mM) and TsOH (0.1 g) in 30 mL benzene were refluxed for 7 hours with Dean-stark azeotropic separation. Workup and recrystallization from hexane gave 2.9 g of a light yellow solid: mp 58 $^{\circ}$ C; 72% yield; NMR (CDCl₃) 10.57 (1H, s, OH), 8.10 (1H, d, J=8.2 Hz, H₅), 7.32 (1H, d, J=2.2 Hz, H₂), 7.13 (1H, dd, J=8.2, 2.2 Hz, H₆), 5.37 (1H, s, acetal), 3.70 (4H, AB q, J_{AB}=11.7 Hz), 1.26 (3H, s, methyl), 0.83 (3H, s, methyl); MS *m/e* 254 (M⁺+1, 100), 235(M-water, 33), 223(M-NO or 2 methyl, 30), 115(30).

2A. # 1 (1.5 g, 5.9 mM) was hydrogenated with 10% Pd/C in ethanol for 4 hours. Filtration and evaporation gave 1.16 g: red solid; mp 285 $^{\circ}$ C; 88% yield, NMR (CDCl₃) 6.89 (1H, d, J=2.2 Hz, H₂), 6.85 (1H, dd, J=8.0, 2.2 Hz, H₆), 6.66 (1H, d, J=8.0 Hz, H₅), 5.17 (1H, s, acetal), 3.68 (4H, AB q, J_{AB}=11.0 Hz), 1.30 (3H, s, methyl), 0.78 (3H, s, methyl); MS *m/e* 224 (M⁺+1, 100), 138 (28), 115 (53).

B. 100 mg Ra-Ni suspension was added to # 1 (1.5 g, 6 mM), 1 mL hydrazine hydrate in 30 mL ethanol and 10 mL water. The reaction was refluxed for 40 min, decanted and worked up to give 0.7 g, light brown solid: 52% yield, identical by NMR to A.

3A. To # 2B (0.7 g, 3.4 mM), were added NaHCO₃ (0.4 g) in 25 mL water and phenyl chloroformate (0.6 g, 3.8 mM) in 25 mL ethanol. After 30 minutes at room temperature, NaOH (0.3 g) in 15 mL water was added. After 0.5 hours HCl was added until acidic pH and the reaction was worked up to give after trituration with hexane 335 mg: mp 191 0 C; 78% yield; NMR (acetone d₆) 9.97 (1H, s, CHO), 7.81 (1H, dd, J=8.0, 2.2 Hz, H₆), 7.72 (1H, d, J=2.2 Hz, H₂), 7.34 (1H, d, J=8.0 Hz, H₅).

4. # 3A (25 mg, 0.155 mM), 2-cyano-3',4'-dihydroxy acetophenone, AG 532 (40 mg, 0.245 mM) (30), and -alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 3 hours, evaporated and purified by HPLC semipreparative RP18 column. # 4 was eluted by 41% acetonitrile in water (containing 0.1% TFA), lyophilized to give 24 mg, of yellow powder: mp 240°C; 48% yield; NMR (acetone-d₆) , 8.08 (2H, s), 7.94 (1H, dd, J=8.2, 1.1 Hz), 7.42 (2H, m), 7.36 (1H, d, J=8.2 Hz), 6.98 (1H, d, J=8.7 Hz); MS *m/e* 321 (M⁻-1, 100).

7. 3-nitro 4-hydroxy benzaldehyde (1.64 g, 10 mM), 1,3-dihydroxy 2,2-dimethyl propane (1.5 g, 14 mM) and TsOH (0.1 g) in 30 mL benzene were refluxed 16 hours with Dean-stark azeotropic separation. Workup and recrystallization from hexane gave 1.55 g, light yellow solid: mp 45 $^{\circ}$ C; 61% yield; NMR (CDCl₃) 10.63 (1H, s, OH), 8.27 (1H, d, J=2.2 Hz, H₂), 7.77 (1H, dd, J=8.8, 2.2 Hz, H₆), 7.16 (1H, d, J=8.8 Hz, H₅), 5.37 (1H, s, acetal), 3.70 (4H, AB q, J_{AB}=11.0 Hz), 1.29 (3H, s, methyl), 0.83 (3H, s, methyl); MS *m/e* 253 (M⁺, 25), 223 (M-NO, 11), 201(M, 100), 186 (22), 177 (14), 132 (15).

Reaction on a larger scale for 6 hours gave 86% yield and for 12 hours 75% yield.

8A. # 7 (1.47 g, 6.7 mM) was hydrogenated with 10% Pd/C in ethanol for 6 hours. Filtration and evaporation gave 0.82 gr, light yellow solid: mp 125 $^{\circ}$ C; 55% yield; NMR (CDCl₃) 6.94 (1H, d, J=2.2 Hz, H₂), 6.80 (1H, dd ,J=8.0, 2.2 Hz, H₆), 6.69 (1H, d, J=8.0 Hz, H₅), 5.27 (1H, s, acetal), 3.61 (4H, AB q ,J_{AB}=11.0 Hz), 1.28 (3H, s, methyl), 0.78 (3H, s, methyl); MS *m/e* 224 (M⁺+1, 100), 138 (32), 115 (46).

B. To # 7 (1.5 gr, 6 mM), and 1 mL hydrazine hydrate in 30 mL ethanol and 10 mL water, 100 mg Ra-Ni suspension was added. The reaction was refluxed 40 minutes, decanted and worked up to give 0.75gr, 58% yield, white solid, identical (TLC, NMR) to # 8A.

9. To # 8A (0.67 g, 3 mM), NaHCO₃ (0.3 g) in 25 mL water and 25 mL ethanol, phenyl chloroformate (0.5 g, 3.2 mM) was added. After 20 minutes NaOH (0.15 g) in 20 mL water was added. After 0.5 hours HCl was added until acidic pH and the reaction worked up to give after trituration from hexane 284 mg: mp 163 $^{\circ}$ C; 69% yield; NMR (CDCl₃) 9.95 (1H, s, CHO), 7.70 (1H, dd, J=8.0, 2.2 Hz, H₆), 7.63 (1H, d, J=2.2 Hz, H₂), 7.35 (1H, d, J=8.0 Hz, H₅); NMR (acetone d₆) 9.99 (1H, s, CHO), 7.77 (1H, dd, J=8.2, 1.9 Hz, H₆), 7.64 (1H, d, J=1.9 Hz, H₂), 7.46 (1H, d, J=8.2 Hz, H₅).

10. # 9 (6 mg, 0.04 mM), AG 532 (7 mg, 0.05 mM) and -alanine (1 mg, 11.23 μ M) in 20 mL ethanol were refluxed 4 hours. Evaporation and trituration from acetonehexane gave 2.5 mg, yellow solid: mp 210 °C; 15% yield; NMR (acetone d₆) 8.06 (1H, s, vinyl), 8.03 (1H, d, J=1.7 Hz), 7.81 (1H, dd, J=8.0, 1.7 Hz), 7.60-6.60 (4H, m). # 13. Anhydrous DMF (4.3 mL, 67 mM), was added dropwise to finely ground AlCl₃ (26.65 g, 0.2 M), with stirring, under argon. The mixture was heated to 45 °C, benzoxazolone (Aldrich, 2.7g, 20 mM) and bromo acetyl bromide (2.65 mL, 30 mM) were added slowly. After 30 minutes the mixture was heated to 95 °C for 4.5 hours, then poured into ice (1 kg), and stirred for 1 hour. The precipitate was filtered and washed with 1 L of water, dried and recrystallized from methanol to give 4.6 g, light brown solid: mp 205 °C; 90% yield; NMR (acetone-d₆) 7.97 (1H, dd, J=8.2, 1.6 Hz), 7.89 (1H, d, J=1.2 Hz), 7.30 (1H, d, J=8.1 Hz), 4.75 (2H, s); MS *m/e*, 254 (M⁻-2), 255 (M⁻-1).

14. # 13 (2g, 7.87 mM), and KCN (1.02 g, 15.7 mM) was dissolved in 10% water/ethanol (400mL). The mixture was stirred and heated to 45° C for 1.5 hours, 200 mL of water were then added and the mixture was titrated to pH 6 with HCl. The mixture was stirred 0.5 hour, then brought to pH=7 with KOH. The ethanol was

evaporated and the product was extracted with 3x200 mL ethyl acetate, evaporated, and chromatography was performed on silica gel (35-70 mesh). # 14 was eluted with 0.6% methanol in dichloromethane to give: 180 mg, light grey solid; mp 200 °C; yield 11%; NMR (acetone-d₆) 7.92 (1H, dd, J=8.2, 1.6 Hz), 7.86 (1H, d, J=1.6 Hz), 7.31 (1H, d, J=8.2 Hz), 4.58 (2H, s); MS *m/e*, 201 (M⁻¹).

15. # 14 (20 mg, 0.1 mM), 3.4 dihydroxy benzaldehyde (13.6 mg, 0.1 mM) and – alanine (1.22 mg, 14 μ M) in 10 mL ethanol were refluxed 4.5 hours, evaporated and purified by HPLC semipreparative RP18 column. The product was eluted by 30% acetonitrile in water, lyophilized to give 12 mg, yellow powder: mp 269 °C; 38% yield; NMR (acetone-d₆) 7.98 (1H, s, vinyl), 7.83 (1H, d, J=2 Hz), 7.78-7.75 (2H, m), 7.47 (1H, dd, J=8.3, 2 Hz), 7.31 (1H, d, J=8.1 Hz), 7.02 (1H, d, J=8.3 Hz); MS *m/e* 323 (M⁺+1, 100), 273 (50).

5. # 14 (31 mg, 0.155 mM), # 10 (25.2 mg, 0.155 mM) and - alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 3 hours, then evaporated. The product was purified by HPLC semipreparative RP18 column, and was eluted by 46% acetonitrile in water, to give 42 mg of light yellow powder: mp 182 °C; 79% yield; NMR (acetoned₆) 8.17 (1H, s, vinyl), 8.09 (1H, d, J=1.5 Hz), 7.96(1H, dd, J=8.3, 1.7 Hz), 7.84 (1H, dd, J=8.1, 1.6 Hz), 7.79 (1H, d, J=1.5 Hz), 7.37 (1H, d, J=8.2 Hz), 7.33 (1H, d, J=8.0 Hz); MS *m/e* 346 (M⁻¹, 100).

11. # 14 (31 mg, 0.155 mM), # 9 (25.2 mg, 0.155 mM) and alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 2.5 hours, then evaporated and purified by HPLC semipreparative RP18 column. The product was eluted by 47% acetonitrile in water, to give 41.8 mg of light yellow powder: mp 187 °C; 77% yield; NMR (acetone-d₆) 8.16 (1H, s, vinyl), 8.04 (1H, d, J=1.8 Hz), 7.84 (3H, m), 7.45 (1H, d, J=8.4 Hz), 7.33 (1H, d, J=8.1 Hz); MS *m/e*, 346 (M⁻¹, 100).

16. 4-hydroxy acetophenone (27 g, 0.2 M) was added to 100 mL glacial acetic acid and 25 mL of 70% nitric acid, with ice cooling and stirring for 6 hours. The reaction mixture was kept overnight at 4° c while crystallization started. 100 mL water were added, the mixture was cooled in crushed ice for 0.5 h, and the precipitate was filtered, washed, and dried. The precipitate was recrystallized from ethanol to give 19.6 g, light yellow solid: mp 118 °C; 54% yield; NMR (DMSO-d₆) 8.41 (1H, d, J=2 Hz), 8.09 (1H, dd, J=8.7, 2.1 Hz), 7.21 (1H, d, J=8.7 Hz), 2.56 (3H, s) (31).

17. # 16 (10 g, 55 mM) was hydrogenated with 10% Pd/C in 150 mL ethanol for 3 hours. Filtering and evaporation gave 7.6 g brown solid: mp 93 $^{\circ}$ C; 92 % yield; NMR (DMSO-d₆) 7.20 (1H, d, J=2.2 Hz), 7.13 (1H, dd, J=8.2, 2.2 Hz), 6.71 (1H, d, J=8.1 Hz), 2.40 (3H, s).

18. # 7 (7.6 g, 50 mM), NaHCO₃ (4.2 g) and phenyl chloroformate (7.8 g) in 150 mL water and 350 mL ethanol were stirred at room temperature for 35 min. NaOH (3.75 g) in 80 mL water was added and the reaction was stirred an additional 30 min. HCl was added slowly until pH 4, the reaction was stirred 10 min and brought back to pH 7 with NaOH 1M. The product was extracted with 3x150 mL ethyl acetate, and then evaporated to dryness. Trituration with ethyl acetate-water, and then with ethanol-water gave 4.94 g, light brown solid: mp 200 $^{\circ}$ C; 56% yield; NMR (DMSO-d₆) 7.83 (1H, dd, J=8.4, 1.8 Hz), 7.57(1H, d, J=1.7 Hz), 7.41 (1H, d, J=8.4 Hz), 2.58 (3H, s).

19. # 18 (4.94 g, 28 mM) and finely ground CuBr₂ (12.5 g, 56 mM) in 150 mL ethyl acetate and 150 mL chloroform were refluxed 15 hours. The reaction mixture was evaporated and workuped. 200 mL water were added, filtering and drying gave 3.9 g of light brown solid: mp 200 $^{\circ}$ C; 55% yield; NMR (acetone-d₆) 7.92 (1H, dd, J=8.4, 1.8 Hz), 7.78 (1H, d, J=1.7 Hz), 7.39 (1H, d, J=8.4 Hz), 4.77 (2H, s); MS *m/e* 258 (M⁺+2, 30), 256 (M⁺, 30), 178 (M-Br, 20).

20. # 19 (1.024 g, 4 mM) and NaCN (0.98 g, 20 mM) in 100 mL ethanol and 20 mL water were heated to 55° c for 25 min. Ethanol was evaporated, 150 mL water were added and the mixture was brought to pH 7 with HCl 1 M (about 5 mL). The product was extracted with 3x100 mL ethyl acetate, washed with water, and purified by HPLC chromatography using preparative RP18 column. The product was eluted by 25% acetonitrile in water and lyophilized to give 235 mg, white powder: mp 230 °C; 29% yield; NMR (acetone-d₆) 7.87 (1H, dd, J=8.4, 1.8 Hz), 7.76 (1H, d, J=1.5 Hz), 7.40 (1H, d, J=8.4 Hz), 4.60 (2H, s); MS *m/e*, 201 (M⁻¹, 100).

21. # 20 (31 mg, 0.155 mM), 3,4-dihydroxy benzaldehyde (21.4 mg, 0.155 mM) and – alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 2.5 hours, evaporated and purified by HPLC semipreparative RP18 column. # 21 was eluted by 42% acetonitrile in water, mp 280 °C to give 28.8 mg of light yellow powder: 58% yield; NMR (acetone-d₆) 7.98 (1H, s, vinyl), 7.82 (1H, d, J=2.2 Hz), 7.70 (1H, dd, J=8.3, 1.8 Hz), 7.60 (1H, d, J=1.4 Hz), 7.47 (1H, dd, J=8.5, 2.2 Hz), 7.40 (1H,d, J=8.3 Hz), 7.01 (1H, d, J=8.3 Hz); MS *m/e* 321 (M⁻¹, 100).

6. # 20 (31 mg, 0.155 mM), # 3 (25.2 mg, 0.155 mM) and - alanine (1.38 mg, 15.5 μ M), in 10 mL ethanol were refluxed 3.5 hours and evaporated to give 51 mg of light yellow powder: mp 288 °C; 96% yield; NMR (acetone-d₆) 8.38 (1H, s, vinyl), 8.29 (1H, d, J=1.7 Hz), 8.15 (1H, dd, J=8.4, 1.7 Hz), 7.96 (1H, dd, J=8.3, 1.8 Hz), 7.86 (1H, d, J=1.7 Hz), 7.62 (1H, d, J=8.3) 7.58 (1H, d, J=8.1); MS m/e, 346 (M⁻-1, 100).

12. # 20 (31 mg, 0.155 mM), # 9 (25.2 mg, 0.155 mM) and -alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 3.5 hours, then evaporated to give 50 mg of light yellow powder: mp 266 °C; 94% yield; NMR (acetone-d₆) 8.17 (1H, s, vinyl), 8.05 (1H, d, J=1.9 Hz), 7.81 (1H, dd, J=8.4, 1.7 Hz), 7.76 (1H, dd, J=8.3, 1.8 Hz), 7.6 (1H, d, J=1.4 Hz), 7.46 (1H, d, J=8.5 Hz) 7.43 (1H, d, J=8.4 Hz); MS *m/e*, 346 (M⁻-1 100).

23. AG 532 (27.3 mg, 0.155 mM), isovanilline (23.5 mg, 0.155 mM) and – alanine (1.38 mg, 15.5 μ M) in 10 mL ethanol were refluxed 2.5 hours, evaporated and purified by HPLC semipreparative RP18 column. Elution with 38% acetonitrile in water gave 15 mg of light yellow powder: mp 110 °C 32% yield; NMR (acetone-d₆) 7.93 (1H, s, vinyl), 7.78 (1H, d, J=2.5 Hz), 7.57 (1H, dd, J=8.5, 2.3 Hz), 7.39 (2H, m), 7.15 (1H, d, J=8.5 Hz), 6.97 (1H, dd, J=7.6, 0.9 Hz), 3.98 (3H, s); MS *m/e* 312 (M⁺+1, 100).

25. AG 538 (51 mg, 0.17 mM) (30), acetic anhydride (3 mL, 32 mM) and 3 drops pyridine, were brought to 100 $^{\circ}$ C for three minutes, then cooled and stirred overnight. 40 mL water were Added, reaction mixture was stirred for 1.5 hours, worked-up and purified by HPLC semipreparative RP18 column. Elution with 43-50% acetonitrile in water, gave 11.6 mg of light yellow powder: mp 48 $^{\circ}$ C 15% yield; NMR (acetone d₆)

8.18 (1 s, vinyl), 8.06 (1H, dd, J=8.1, 1.7 Hz), 8.01 (1H, d, J=2.1 Hz), 7.92 (1H, dd, J=8.4, 2.1 Hz), 7.85 (1H, d, J=2 Hz), 7.52 (1H, d, J=8.5 Hz), 7.49 (1H, d, J=8.4 Hz), 2.33 (9H, M), 2.31(3H, S), MS *m/e* 487 (M⁺+Na, 100).

26. 4-hydroxy-3 methoxy Acetophenone (1 g, 6 mM) and finely ground CuBr₂ (3.35 g, 15 mM) in 40 mL ethyl acetate and 40 mL chloroform were refluxed 18 hours. The reaction mixture was filtered, evaporated and worked-up. Chromatography was performed on silica (35-70 mesh). # 26 was eluted with dichloromethane to give 1.06 g of white solid: mp 55 $^{\circ}$ C; 72% yield; NMR (acetone-d₆) 8.63 (1H, s, OH), 7.65 (1H, d, J=2, J=8.3 Hz), 7.59 (1H, d, J=2 Hz), 6.95 (1H, d, J=8.3 Hz), 4.66 (2H, s), 3.93 (3H, s); MS *m/e* 166 (M-Br, 100), 247 (M⁺+1, 75).

27. # 26 (0.71 g, 2.9 mM) and NaCN (0.71 g, 14.5 mM) in 100 mL ethanol and 20 mL water were heated to 55° c for 30 min. Ethanol was evaporated, 100 mL water were added and the mixture was brought to pH 7 with HCl 1 M (about 10 mL). The product was extracted with 3x100 mL ethyl acetate, washed with water, and purified by HPLC

chromatography using preparative RP18 column. The product was eluted by 28% acetonitrile in water and lyophilized to give 112 mg, white powder: mp 150 °C; 20.5% yield; NMR (CDCl₃) 7.53 (1H, d, J=2 Hz), 7.44 (1H, dd, J=2, J=8.3 Hz), 6.98 (1H, d, J=8.3 Hz), 6.22 (1H, s, OH), 4.02 (2H, s), 3.98 (3H, s); MS *m/e*, 190 (M⁻-1, 100). # 28. # 9 (25 mg, 0.16 mM), #27 (29 mg, 0.15 mM) and -alanine (1.4 mg, 15 μ M) in 10 mL ethanol were refluxed 3 hours and then evaporated. The product was purified by HPLC using a preparative RP18 column, and was eluted by 44% acetonitrile in water, to give 13.2 mg of yellow powder: mp 257 °C; 44 % yield; NMR (acetone-d₆) 8.09 (1H, s, vinyl), 8.04 (1H, d, J=1.7 Hz), 7.82 (1H, dd, J=8.4, 1.7 Hz), 7.55 (2H, m), 7.45 (1H, d, J=8.3 Hz), 6.99 (1H, d, J=8.0 Hz), 3.9 (3H, s); MS *m/e* 337 (M⁺1, 100). # 29. #27 (29.5 mg, 0.155 mM), 3,4-dihydroxy benzaldehyde (21.3 mg, 0.155 mM) and -alanine (1.38 mg, 15.5 µM) in 10 mL ethanol were refluxed 3.5 hours, evaporated and purified by HPLC preparative RP18 column. Elution with 40% acetonitrile in water gave 36 mg of light yellow powder: mp 219 °C 75 % yield; NMR (acetone-d₆) 7.93 (1H, s, vinyl), 7.82 (1H, d, J=2.2 Hz), 7.48 (3H, m), 6.99 (2H, m), 3.95 (3H, s); MS *m/e* 312 (M⁺+1, 100).

RESULTS

Inhibition of IGF-1R and of other kinases in cell free assays

AG 538 bioisosteres and other analogs were prepared by the Knoevenagel condensation of an aldehyde with a derivative of cyano acetophenone. The products were purified by HPLC using a preparative RP18 column, then lyophilized and analyzed by ¹H-NMR and LC-MS, as described in the Experimental Procedures section.

The efficacy of the compounds as kinase inhibitors was examined by ELISA assays. Phosphorylation of pGT by the IGF-1R, IR or Src was performed in the presence or absence of inhibitor. Tyrosine phosphorylated pGT was detected by binding to anti phosphotyrosine antibody then HRP-secondary antibody followed by a color reaction as described in the Experimental Procedures section (Src ELISA assay is described in (32)). PKB inhibition was examined by a radioactive assay, in which the RPRTSSF peptide was phosphorylated by PKB in the presence or absence of inhibitor, as described (33).

The IC₅₀ values of these compounds were compared to the IC₅₀ of AG 538 as shown in Table 1. Substitution of the catechol ring in # 23 and # 29 with a methoxy catechol group reduced the potency of the inhibitor 25 and 55 fold respectively, towards IGF-1R. Acetylating all 4 hydroxyls in # 25 totally destroyed potency, while replacing one of the catechol rings with a benzoxazolone ring (# 4, # 10) reduced the inhibition only by 6-7 fold. We found minor differences between the two isomers # 4 and # 10. In # 21, substitution of the catechol ring with a benzoxazolone on the other side of the molecule reduced the inhibition by 10 fold as compared to AG 538. Replacing both catechol rings with a benzoxazolone reduced markedly the activity of the compounds (# 5, # 6, # 11

and # 12). # 4 and # 10 inhibited other kinases than IGF-1R, with reduced efficacy, similarly to AG 538.

Mechanism of inhibition of IGF-1R by # 10 and # 4

The mechanism of inhibition of IGF-1R was tested for the two most active molecules: # 10 and # 4. Table 2 shows that the IC_{50} for inhibition of IGF-1R substrate phosphorylation by # 10 is independent of ATP over a wide concentration range, suggesting that # 10 is non-competitive vis-à-vis ATP. The use of the radioactive assay (described in the Experimental Procedures) allowed us to perform kinetic analysis of IGF-1R inhibition by # 4 and # 10. Analyzing the data with a Lineweaver-Burk plot demonstrated that # 4 inhibited the IGF-1R in a substrate competitive manner (Figure 1). # 10 was also found to inhibit the IGF-1R in the same manner (data not shown).

Inhibition of IGF-1R autophosphorylation and downstream signaling in intact cells To assay inhibition of IGF-1R autophosphorylation and of the downstream elements, IRS-1, PKB and Erk-2 in intact cells, AG 538, # 4 and # 10 were incubated with NWTc43 cells for 17 hours as described in the Experimental Procedures section. Gels were blotted and probed with anti phosphotyrosine 4G10 antibody (Figure 2A) anti phospho-IRS-1 antibody (Figure 2B), anti phospho-Akt antibody (Figure 2C), and anti phospho-Erk antibody (Figure 2D). IGF-1R autophosphorylation, activation of IRS-1, Erk and PKB/Akt were all inhibited by AG 538, # 10 and # 4, in a dose dependent manner over a similar concentration range (Figure 2). Inhibition of IGF-1R downstream signaling, i.e. the phosphorylation of IRS-1, PKB and Erk-2, was also detected after a short incubation of 40 minutes with the inhibitors with a similar dose dependency (data not shown).

Inhibition of IGF-1 induced PKB activation in intact cells

AG 538, # 4 and # 10 caused pronounced inhibition of IGF-1 induced PKB/Akt phosphorylation. We determined the IC_{50} values of these inhibitors after overnight incubation of NWTC43 cells with the inhibitors, as described in the Experimental Procedures section (Figure 3). Gels were blotted and probed with anti phospho-Akt antibody, then stripped and re-probed with anti AKT1/2. # 4 and # 10 inhibited PKB activation more efficiently, at lower concentrations than AG 538. The IC_{50} of # 4 is 12µM, that of # 10 is 6µM and that of AG 538 is 19µM (Figure 3).

PKB was activated by PDGF as described in the Experimental Procedures section. The effect of the inhibitors AG 538, # 4 and # 10 on PDGF induced activation was assayed at 5μ M and 20μ M inhibitors concentrations. No inhibition of PDGF induced PKB activation was detected, as shown in Figure 3D.

Growth inhibition of R⁺, MDA MB-468 and MCF-7 cells

MDA MB-468 and MCF-7 are breast cancer derived cell lines. R^+ cells (described above), MDA MB-468 and MCF-7 cells were sparsely seeded, and one day after seeding the cells were incubated with AG 538 or # 10 or # 4 at various concentrations in growth media for 72 hours. The medium was replaced every 24 hours, and fresh inhibitor was added to the appropriate samples. Figure 4 shows the growth inhibition of R^+ cells by # 10. # 10 and # 4 inhibited the growth of R^+ , MDA MB-468 and MCF-7 cells, at a similar concentration range as AG 538, as shown in Table 3.

Inhibition of colony formation in soft agar

MDA MB-468, PC-3, LNCaP and MCF-7 cells were seeded in agar, one day later inhibitors were added at various concentrations. After 7-12 days, colonies were stained with MTT, counted and photographed. Figure 5 shows that treatment with 25 μ M and 30 μ M inhibitors blocked colony formation in soft agar almost completely in MDA MB-468 and PC-3 cells. Table 4 summarizes the IC₅₀ values for the inhibition of colony formation by the inhibitors, on various cell lines.

Inhibition of IGF-1R signaling in breast cancer and prostate cancer cell lines

The IGF-1R blockers AG 538, # 4 and # 10 inhibit colony formation in soft agar of two breast and two prostate cancer cells. The inhibitors were examined for their ability to inhibit the IGF-1R signaling in MDA MB-468 (breast cancer cells) and PC-3 (prostate cell lines). The IGF-1 induced tyrosine phosphorylation of IRS-1 was brought to a lesser degree than maximal activity, at 10 μ M inhibitor, where at 50 μ M the extent of IRS-1 was below basal as shown in Figure 6. The signal of PKB was below detection levels.

DISCUSSION

The catechol moiety mimics the phenol group of tyrosine, and therefore many tyrosine kinase inhibitors from the tyrphostin family contain a catechol moiety. It is well known that the catechol moiety is oxidized fairly rapidly, rendering catechol compounds unstable (19). In order to improve the stability of catechol compounds, bioisosteres were developed (# 4, # 5, # 6, # 10, # 11, # 12, # 15, # 21 and # 28).

Attempts to alter the catechol moieties of AG 538 caused reduction in potency: Substitution of the catechol ring in # 23 and #29 with a methoxy group reduced the potency of the inhibitor 25 and 55 fold respectively, and acetylation of the hydroxyl group (# 25) totally abolished inhibition (Table 1). In this report we describe the synthesis of eight new AG 538 analogs that possess a benzoxazolone group in place of the catechol moiety on one or both sides of AG 538. Compounds that possess one benzoxazolone ring, namely # 4, # 10, # 15 and # 21, inhibit the IGF-1R, selectively, and do not inhibit Src and PKB (Table 1). Two of the more potent compounds, # 4 and # 10, were chosen for further investigation. These inhibitors are 6-7 fold less active as compared to AG 538. AG 538 as well as its bioisostere analogs are selective for IGF-1R, as compared to their inhibitory activity towards Src and PKB in cell free assays (Table 1) and towards the PDGFR signaling pathway in intact cells (Figure 3D).

Both # 4 and # 10 are substrate competitive inhibitors of the IGF-1R (Figure 1 and data not shown). Such inhibitors may have an advantage over ATP-mimicking inhibitors that have to compete with high intracellular ATP concentrations (for review see (34)).

The IGF-1R is known to activate two major pathways, the anti-apoptotic pathway mediated by PI3K-PKB and the Shc-Ras–Erk proliferative pathway. The IRS proteins are direct substrates of the IGF-1R and mediate the PI3K-PKB pathway as well as others. When applied to cells, # 4 and # 10 inhibit IGF-1R autophosphorylation as

well as IGF-1R induced phosphorylation of IRS-1, PKB and Erk2, in a dose responsive manner, similarly to AG 538 (Figure 2). A shorter (40 minutes) incubation of these inhibitors in intact cells also inhibits IGF-1R downstream signaling i.e. phosphorylation of IRS-1, PKB and Erk-2, in a dose responsive manner (data not shown) For the compounds tested, the concentrations needed to inhibit the autophosphorylation of IGF-1R were higher than the concentrations needed to inhibit PKB and IRS-1. This difference may result because autophosphorylation represents intermolecular transautophosphorylation within the IGF-1R dimer. Therefore the inhibitor must compete against a local substrate concentration that is much higher than the concentrations of exogenous substrates like IRS-1 or PI-3 kinase.

The effect of the inhibitors on IGF-1-induced Erk2 phosphorylation in cells is less pronounced than the effect on PKB and IRS-1 phosphorylation (Figure 2). Erk2 activation is mediated by Shc protein, a separate signaling pathway from IRS-1/ PI-3 kinase/ PKB (35). Since AG 538, # 4 and # 10 are IGF-1R substrate competitive, the affinity of Shc proteins to IGF-1R and their concentrations in the cells should determine the efficacy of the inhibitors towards the Shc protein pathway (Erk2), whereas the affinity of IRS-1 to IGF-1R and the concentration of IRS-1 should determine the efficacy towards the IRS-1/ PKB pathway. The different effects of the inhibitors on IGF-1-induced Erk2 and IRS-1/PKB phosphorylation may derive from differences in the affinities and/or concentrations of the relevant IGF-1R substrates.

Compounds # 4, # 10 and AG 538 inhibit the activation of PKB by the IGF-1R in intact cells, with IC_{50} values of 12µM, 6µM and 19µM respectively. These inhibitors do not affect PKB activation by PDGF-R in intact cells as shown in Figure 3, confirming their selectivity.

Although the cell-free potency of # 4 and # 10 is 6 to 7-fold lower than that of AG 538 (IGF-1R inhibition, Table 1), their potency in cells is similar to that of AG 538, when examined in long-term assays of inhibition of growth and of colony formation in soft agar as shown in Tables 3 and 4. This most likely reflects the increased stability of the bezoxazolone containing compounds in cells.

Inhibitors of the IGF-1R and its signaling pathways could be extremely useful as antineoplastic agents. Indeed the compounds described in this paper inhibit anchorage dependent growth of R^+ and MDA MB-468 breast cancer cell lines (Figure 4 and Table 3). These compounds also led to morphological reversion of transformed R^+ cells (IGF-1R over-expressors), leading to a morphology similar to that of the non-transformed, R^- (IGF-1R deleted) cells (data not shown).

The formation of colonies in soft agar by cancer cells is an indication of their invasiveness, and the IGF-1R is known to be a positive regulator of this phenotype (36,37). Compounds # 4, # 10 and AG 538 were found to inhibit the formation of colonies in soft agar in two breast cancer cell lines and two prostate cancer cell lines (Figure 5 and data not shown). The IC₅₀ values of these inhibitors are presented in Table 4. It can be seen that compounds # 4, # 10 and AG 538 inhibit IRS-1 phosphorylation/activation by IGF-1 in representative breast and prostate cancer cell lines (Figure 6).

Compounds # 4, # 10 and AG 538 inhibit anchorage independent cell growth of the MDA MB-468 and MCF-7 breast cancer lines at lower concentrations than the inhibition of anchorage dependent cell growth. For example, comparison of unanchored to anchored growth inhibition in MDA MB-468 cells: IC_{50} of 8 μ M, 10 μ M or 6 μ M versus IC_{50} of 51 μ M, 38 μ M or 25 μ M respectively. This finding supports the view that IGF-1R may be more important in maintaining the transformed

phenotype than in promoting monolayer cell growth as suggested by (38). This study describes the action of antisense IGF-1R RNA on human melanoma cells incubated subcutaneously or grown in soft agar or in monolayer. The antisense induced massive apoptosis of the subcutaneous incubated cells, whereas the same cells grown in monolayer treated with the same antisense were only moderately inhibited, and the effect on cells growing in soft agar was somewhere in between. It seems therefore that when cells grow without anchorage, they depend for their survival on anti-apoptotic pathways such as the IGF-1R mediated pathways. Therefore inhibition of the IGF-1R in unanchored cells causes massive apoptosis (39).

The ability of the IGF-1R inhibitors to inhibit anchorage independent cell growth better than anchorage dependent growth has important implications for the toxicity of an IGF-1R inhibitor as a potential therapeutic agent. An agent that can selectively inhibit transformed, unanchored cells without inhibiting normal, anchored cells would be a highly valuable anti tumor drug, since it is likely to possess less toxicity. In summary, in this study, we present a new class of substrate competitive IGF-1R inhibitors, which possess a novel chemical moiety, resistant to oxidation. The ability to replace the catechol groups in substrate competitive tyrphostins with a moiety that is more stable in cells should aid in developing non-catechol tyrosine kinas inhibitor drugs for clinical use. Furthermore, development of bioisostere tyrphostins will be necessary in order to generate potent and selective IGF-1R kinase inhibitors with anti tumor activity.

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FOOTNOTES

Abbreviations

AEBSF	4 (2-aminoethyl)-benzene sulfonyl fluoride
BSA	Bovine serum albumin
HRP	Horseradish peroxidase
IGF-1R	Insulin-like growth factor-1 receptor
IRS-1	Insulin receptor substrate-1
PDGF-R	Platelet derived growth factor receptor
pGT	poly (Glu, Tyr) 4:1
PBS	Phosphate- buffered saline
TBS	Tris-buffered saline
TBST	TBS with 0.2% tween-20

LEGENDS TO FIGURES

Figure 1. Kinetics of inhibition of the IGF-1R by #4

Poly-GT was phosphorylated by semi purified IGF-1R, and IGF-1R activity was determined by the radioactive assay described in Experimental Procedures. IGF-1R was assayed at concentrations of pGT ranging from 0 to 24 μ g/ml and # 4 ranging from 0 to 6 μ M. The data were analyzed by the Lineweaver-Burk plot using the excel program.

Figure 2. Inhibition of IGF-1R signaling

NIH-3T3 cells overexpessing the IGF-1R, were incubated overnight with the indicated inhibitors, followed by one-hour starvation in the presence of inhibitors and short activation with IGF-1. Cells were lysed and equal amounts of protein separated by SDS-PAGE and transferred to a nitrocellulose membrane.

- A. Inhibition of IGF-1R autophosphorylation . Western blot using -phosphotyrosine 4G10 antibody. ii. Reprobing of stripped blot with -IGF-1Rß antibody. iii. The phosphorylation level of each sample (shown in figure Ai) was normalized to the IGF-1R level shown in Aii. These values are shown as percentages of the level of autophosphorylation in the absence of inhibitor (designated 100%).
- B. Inhibition of IRS-1 phosphorylation i. Western blot using phosphotyrosin-IRS-1 antibody.

ii. Reprobing of stripped blot with – IRS-1 antibody. iii. Percent of maximum phosphorylation for each sample was defined in the same manner described in Aiii, except the intensities of the bands obtained with – phosphotyrosin-IRS-1 were normalized to the intensities of the – IRS-1 bands.

C. Inhibition of PKB phosphorylation i. Western blot using – phospho AKT (Thr-308) antibody.

ii. Reprobing of stripped blot with – PKB/Akt1/2 antibody. iii. Percent of maximum phosphorylation for each sample was defined in the same manner described in Aiii, with normalization of phospho-PKB to total Akt1/2.

D. Inhibition of Erk2 phosphorylation i. Western blot using anti D-P-Erk1&2 antibody. ii. Reprobing of stripped blot with – Erk2 antibody iii. Percent of maximum phosphorylation for each sample was defined in the same manner described in Aiii, with normalization of phospho-Erk to total Erk2

Figure 3. Inhibition of IGF-1 induced PKB activation

- A-C, IC_{50} values were calculated using data obtained by a similar procedure to the described in Fig. 2C i-iii. IC_{50} curves and values are shown on the right.
- D. Activation of PKB with PDGF in NIH 3T3 cells, in the presence of inhibitors as indicated, performed as described in Figure. 2.

<u>Figure 4</u>. Growth inhibition by # 10

 R^+ cells were grown in 10% FCS in the presence or absence inhibitor for the indicated number of days. Medium with or without inhibitor was replaced every day, if not indicated differently. Cells density was determined by the Methylene Blue method. The IC₅₀ values are summarized in Table 3.

<u>Figure 5</u>. Inhibition of colony formation of MDA-MB-468 cells and PC-3 cells in soft agar

A suspension of separated A, B. MDA MB-468 and C, D. PC-3 cells was plated in growth medium containing agar. One day later, growth medium supplemented with inhibitors at appropriate concentrations was added. 7-12 days after plating, colonies were stained with MTT, photographed and counted. The IC_{50} of the each inhibitor was calculated. A, C. A representative picture of cells treated with that inhibitor at 25 and 30 μ M respectively. B, D. Inhibition of colony formation of cell after incubation with various concentrations of the inhibitors indicated. The assays were performed in triplicate.

Figure 6. Inhibition of IGF-1R signaling in MDA MB-468 and PC-3 cells

A. 40% confluent MDA MB-468 cells were incubated with the indicated inhibitors for 5 hours in starvation medium followed by a short activation with IGF-1. B. 70% confluent PC-3 cells were incubated with the indicated inhibitors for 17 hours in growth medium followed by a short activation with IGF-1. In both A and B the cells were then lysed and equal amounts of protein separated by SDS-PAGE and transferred to a nitrocellulose membrane. In Ai,ii and Bi,ii blots from a representative experiment are shown. Parts Aiii and Biii show quantification and error bars of two separate experiments are presented in Aiii and Biii.

Inhibition of IRS-1 phosphorylation i. Western blot using – phosphotyrosine-IRS-1 antibody.

ii. Reprobing of stripped blot with -IRS-1 antibody. iii. The intensities of the bands obtained with - phosphotyrosine-IRS-1 were normalized to the intensities of the

- IRS-1 bands. These values are shown as percentages of the level of IRS-1 phosphorylation in the absence of inhibitor (designated 100%).

TABLES

<u>Table 1.</u>

		IC ₅₀ Values [µM]			
COMPOUNDS	NAME	IGF-1R	IR	SRC	PKB
HO CN OH	AG 538	0.06±0.02	0.12±0.03	2±1	76±10
	#4 (GB19)	0.37±0.2	0.6±0.4	1.5±0.4	21±1
	#10 (AGL 2263)	0.43±0.2	0.4±1.6	2.2±0.4	55±10
	#15 (GB16)	1.6±0.8	0.6±0.2	3.1±0.1	~50
	#21 (GB20)	0.6±0.2	0.6±0.1	7±1	~100
	#11 (GB17)	19±7	15±2	50±20	>100
	#5 (GB18)	13.7±5	5.5±0.5	35±10	>100
	#12 (GB21)	32±5	28±4	80±20	>100
	#6 (GB22)	21±5	18±2	80±20	>50
	#23 (GB27)	1.5±1	1.9±0.7	2.5±0.8	~100
	#29 (GB44)	3.5±2	5.2±0.5	-	-
	#28 (GB42)	59±5	60±3	-	-
$H_{3}C \longrightarrow O \longrightarrow C^{+}$	H₃ #25 (GB2) `CH₃	>100	-	-	-

INHIBITION OF IGF-1R AND OTHER KINASES BY AG 538 BIOISOSTERES AND ANALOGS

Table 2. Inhibition of IGF-1R activity by # 10 is not ATP competitive

ATP [µM]	IC ₅₀ [µM]
2	670 +/- 150
6	620 +/- 290
15	880 +/- 200
30	880 +/- 120
60	720 +/- 160
100	710 +/- 60

Table 3. Growth inhibition by AG 538, #10 and #4

	IC ₅₀ [μM]			
INHIBITOR CELL LINE	AG 538	# 10	# 4	
R ⁺	27	36	23	
MDA MB-468	25	38	51	
MCF-7	79	>100	>100	

<u>Table 4.</u> IC_{50} of colony formation in soft agar

INHIBITOR	AG 538	# 10	#4	
CELL LINE	IC ₅₀ [μM]			
PC-3	4.3±0.2	9±3	11±3	
LNCaP	9±4	15.7±0.2	16±2	
MDA MB-468	6±2	10±3	8±2	
MCF-7	17±6	11*	11±1	

* Assay performed once

Scheme 1



Scheme 2







Scheme 3



20

Scheme 4



Scheme 5





Figure 1 Blum et al.

А





С

D



Figure 2 Blum et al.





PKB phosphorylation activated by PDGF





Figure 4 Blum et al.

A. MDA MB-468 CELLS

NO TREATMENT









25µM # 4



B.



Figure 5 Blum et al.

PC-3 CELLS

NO TREATMENT

VEHICHLE



30µM AG 538



30µM # 10



D.

C.





Figure 5 Blum et al.

A. B. PC-3 cells MDA MB-468 i i pIRS-1 pIRS-1 ii ii IRS-1 IRS-1 120 140 pIRS-1/IRS-1 iii iii pIRS-1/IRS-1 120 100 100 80 Ŧ 80 ГŢ T_ 60 60 Т 40 40 20 20 0 00 IGF-1 +++++++IGF-1 +++++++10 50 10 50 AG 538 [µM] AG 538 [µM] _ _ _ _ _ _ _ _ 10 50 10 50 #4 [µM] #4 [µM] _ _ _ _ _ _ _ _ # 10 [µM] 10 50 # 10 [µM] 10 50 _ _

Figure 6 Blum et al.