Effects of the ErbB1/ErbB2 kinase inhibitor GW2974 on androgen-independent prostate cancer PC-3 cell line growth and NSE, chromogranin A and osteopontin content

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Abstract. Prostate cancer is one of the most frequently diagnosed cancer in men. Treatment by radical prostatectomy, radiotherapy and anti-androgen drugs is successful in patients with localized cancer. However, prolonged androgen deprivation often leads to hormone refractory condition, associated with disease relapse. ErbB1 and ErbB2 activity has been correlated with androgen-independence. We determined the effects of GW2974, a dual inhibitor of ErbB-1 and ErbB-2 tyrosine kinase activity, on growth, NSE, chromogranin A and osteopontin cytosol content in the androgen-independent prostate cancer cell line PC-3. We found that PC-3 cell growth was inhibited by GW2974, whereas NSE and chromogranin A cell contents were stimulated and osteopontin cytosol level was not affected. The present data may have clinical implications for the treatment of advanced prostate cancer.

Introduction

Prostate cancer represents one of the most common malignancies diagnosed in the adult men. Patients with a localized tumour are successfully treated by radical prostatectomy, radiotherapy and anti-androgen therapy. Patients with metastatic prostate cancer often develop hormone refractory state together with uncontrolled spread and high mortality rate. A subset of advanced prostate cancer is also characterized by extensive neuroendocrine (NE) differentiation associated with androgen-independence and preferably called castration-resistant prostate cancer (CRPC) (1). At present, there are no effective therapies for CRPC, reflecting poor understanding of what leads progression to androgen independence. It has been hypothesized that this biological evolution may be due to increased growth factor signalling activity (2). Particularly, the epidermal growth factor receptor (EGFR) family members play a remarkable role in this progression. The human EGFR family consists of four closely related transmembrane glycoproteins: EGFR (ErbB1), Her2/neu (ErbB2), Her3 (ErbB-3) and Her4 (ErbB-4) (3). ErbB2 is also referred as c-erbB2, it is structurally and functionally related to the v-erbB retroviral oncogene and has intrinsic tyrosine kinase activities (4). Overexpression of ErbB1 and ErbB2 is associated with poor prognosis, shorter survival and increased rate of relapse (5). These data provide the basis to investigate EGFR-targeted agents on CRPC. Interestingly, selective inhibitor of ErbB1/ErbB2 tyrosine kinase activity, such as Lapatinib (Glaxo-Smithkline) has been developed. Lapatinib (GW572016) was approved by the USA Food and Drug Administration in 2007 for metastatic ErbB2-positive breast cancer patients (6). However, its efficacy in advanced prostate cancer treatment at present is still unclear. Some authors showed that Lapatinib potently inhibited cell growth in the human recurrent prostate cancer cell line CWR-R1 and in LNCaP androgen-dependent cell line (7,8). Moreover, it has been described that EGFR inhibition prevents NE differentiation in androgen-independent prostate cancer cells DU145 (9), whereas it induces NE differentiation in androgen-sensitive LNCaP cell line (10). As it concerns malignant phenotype, EGFR activation can induce osteopontin gene expression (11). Osteopontin is an extracellular matrix protein which binds to integrins and contribute to invasive behaviour of metastatic cells in a variety of malignant cells such as breast and prostate cancer cells (12).

Since ErbB1/ErbB2-targeting drugs represent novel highly promising therapies in cancer patients (13), there is a need to assess biological effects of these agents in an experimental model. In this study, we determined the effects of GW2974 (Sigma), a lapatinib-like agent able to selectively inhibit

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ErbB-1/ErbB2 tyrosine kinase (14), on PC-3 cell growth and on NE-related markers NSE and chromogranin A cell content. Moreover, we quantified cytosol osteopontin amount of treated and control cells to evaluate the consequence of GW2974 exposure on PC-3 metastatic potential.

Materials and methods

Cell culture. Androgen-independent PC-3 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown at 37˚C in DMEM media supplemented with 10% fetal bovine serum (v/v) (Invitrogen), 1% glutamine (Sigma Aldrich, St. louis, MO) and antibiotics (100 units of penicillin and 100 μg of streptomycin). Cells were treated with several concentrations of GW2974 (Sigma Aldrich). As a control, cells were treated with DMSO, the solvent for GW2974.

Assay for cell proliferation. For growth assay, cells were seeded on 96-well plates at 2-3x10⁴/well in 200 μl medium and synchronized in cell cycle G_0 phase by culture in serum-free medium for about 12 h. Then, the medium was replaced with DMEM containing 10% FBS and GW2974 at 1, 2, 3 and 4 μM. In the control wells an equal amount of DMSO vehicle was added. After 48 h, the rate of cell growth was evaluated by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) colorimetric assay. This assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetazolium rings of the yellow MTT and form blue crystals which are largely impermeable to cell membranes (15). Each single GW2974 dose was done in triplicate in each assay. Percent of growth was normalized to control (untreated) cells (100% growth). The results shown are the mean of three independent experiments.

Cytosol preparation. The cells were seeded at 20x10⁴/well in a 6-well plates in incubation medium (DMEM-10% FBS with antibiotics). After 24 h the medium of each well was replaced with serum-free medium and cells were starved for 12 h. Then fresh incubation medium containing GW2974 at 0.1, 1 and 2 μM was added. In the control wells an equal amount of DMSO vehicle was added.

After 48 h, the medium was removed, the cells were washed with phosphate-buffered saline solution and scraped in ice, then subjected to five strokes of homogenization in buffer (10 mM Tris HCl at pH 7.4, 50 mM NaCl, 1% aprotinin, 1% PMSF and 5 mM EDTA). The homogenate was centrifuged for 10 min at 1500 x g, the supernatant was divided in aliquots and stored at -20˚C until used to quantify NSE, chromogranin A and osteopontin or solubilized in 1X Laemmli buffer for immunoblotting procedure. Proteins were determined by the Bradford procedure.

Determinations of NSE, chromogranin A and osteopontin cytosol content. The amount of NSE, chromogranin A and osteopontin in PC-3 cytosol was determined by ELISA kit (Pantec, Torino, Italy). The data were calculated as ng per well. Results were expressed as percent of control samples (100%). Each experiment was repeated three times.
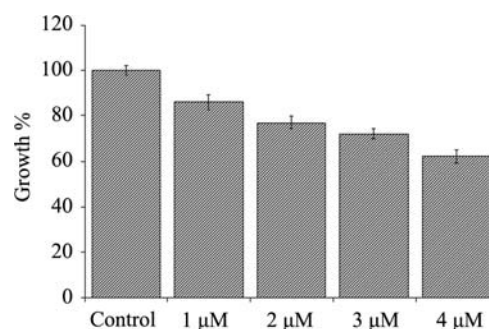

Figure 1. Effect of GW2974 on cell growth measured after 48-h treatment with GW2974 from 1 to 4 μM compared with non-treated cells (control). Data obtained from three experiments are showed as mean with error bars representing standard deviation. P<0.05 was considered significant.

Immunoblotting of NSE to PC-3 cytosol. Cytosols from PC-3 were resolved by 10% SDS-PAGE under reducing condition. After electrophoresis, samples were transferred from gels to nitrocellulose sheets and incubated with non-fat dry milk in Tris-buffered saline at 20˚C for 2 h and processed by adding the antibody anti-NSE (Dako, Milan, Italy) or anti-Actin (Sigma Aldrich) for 1 h at room temperature. Antibodies were detected by a secondary biotinylated antibody and SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology).

For reprobing, the nitrocellulose sheets were stripped using Restore Western blotting stripping buffer as recommended by the manufacturer (Pierce Biotechnology).

Statistical analysis. Differences between control and treated cells were assessed using the Wilcoxon-Mann-Whitney test. P-values of <0.05 were considered significant.

Results

To study the effects of ErbB1 and ErbB2 pathway inhibition in CRPC, we used PC-3, an highly malignant cell line established from bone metastasis, as an *in vitro* model. Importantly, this cell line does not express androgen receptor and PSA and is not responsive to androgen treatment (16). As NE-related markers, PC-3 expresses NSE and chromogranin A (17,18).

Effect of GW2974 on PC-3 cell growth. To determine the role of ErbB1 and ErbB2 receptors activity in androgen-independent prostate cancer cell proliferation, we treated PC-3 cells with different concentrations of GW2974. For each drug concentration the MTT assay was performed in three independent experiments and the relative growth rates were calculated comparing the data with PC-3 vehicle-treated control cells cultured in medium without GW2974. The growth of PC-3 cells was significantly reduced (P<0.05) by each individual GW2974 dose with an inhibitory effect of about 14% at 1 μM, 23% at 2 μM, 28% at 3 μM and 38% at 4 μM (Fig. 1).

Effect of GW2974 on NSE cytosol content. To evaluate the influence of ErbB1 and ErbB2 tyrosine kinase activity on NE phenotype, we examined the effect of GW2974 on PC-3
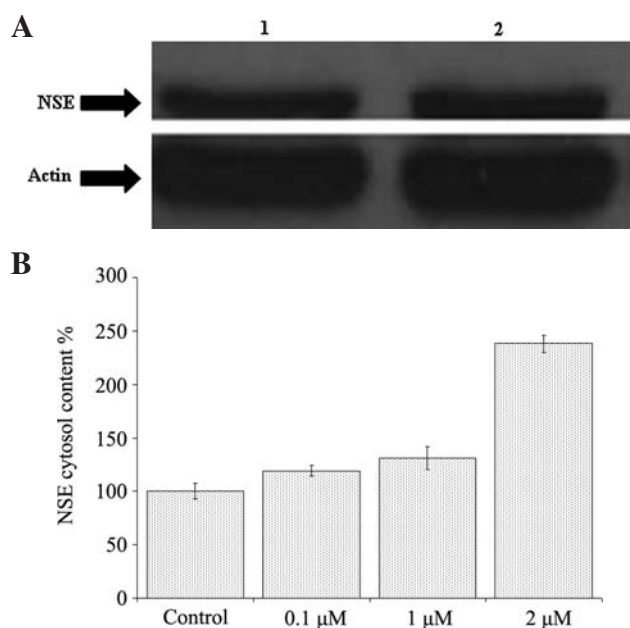
Figure 2. (A) Immunoblot showing stimulatory effect of GW2974 on NSE cytosol content in PC-3 cells (lane 1, control; lane 2, 48 h GW2974 1 μM); (B) Effect of GW2974 on NSE cytosol content after 48-h treatment with GW2974 from 0.1 to 2 μM compared with non-treated cells (control). Data obtained from three experiments are presented as mean with error bars representing standard deviation. P<0.05 was considered significant.

Figure 3. Effect of GW2974 on chromogranin A cytosol content after 48-h treatment with GW2974 from 0.1 to 2 μM compared with non-treated cells (control). Data obtained from three experiments are presented as mean with error bars representing standard deviation. P<0.05 was considered significant.

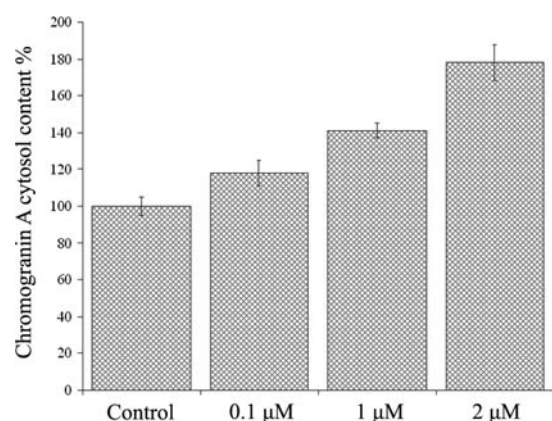cytosol NSE content by Western blot analysis. In Fig. 2A we report a typical experiment showing that NSE cell content increased in GW2974-treated cells. Equal loading of protein in all samples was confirmed by the exposure of the stripped nitrocellulose sheet to actin antibody. This result was confirmed by ELISA assay. In PC-3 cells treated with each individual GW2974 concentration, NSE cytosol content was significantly increased (P<0.05). Mean value compared with control (6.38±0.45 ng/well) was slightly higher at 0.1 μM (7.60±0.40 ng/well) and at 1 μM (8.38±0.95 ng/well), whereas it was >2-fold higher at 2 μM (15.20±1.21 ng/well) (Fig. 2B).
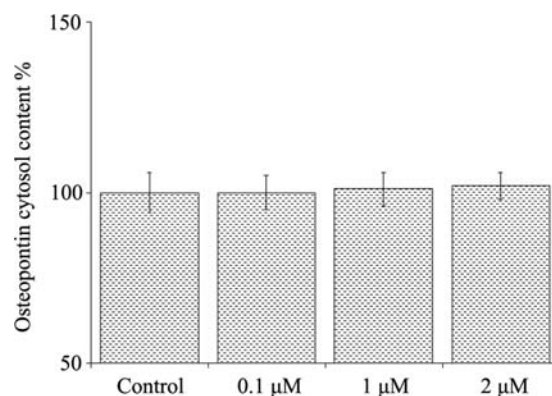

Figure 4. Effect of GW2974 on osteopontin cytosol content after 48-h treatment with GW2974 from 0.1 to 2 μM compared with non-treated cells (control). Data obtained from three experiments are presented as mean with error bars representing standard deviation. P<0.05 was considered significant.

Effect of GW2974 on chromogranin A cytosol content. We also evaluated the effect of GW2974 on PC-3 cytosol chromogranin A by ELISA test. As shown in Fig. 3, chromogranin A levels were significantly increased (P<0.05) by adding each single dose of the dual ErbB1/ErbB2 inhibitor to PC-3 cell culture medium. However, the content of this marker was about 10-fold lower than NSE one. Chromogranin A mean level was weakly higher in PC-3 cells treated with 0.1 μM (0.44±0.03 ng/well) and 1 μM (0.53±0.02 ng/well) and nearly 2-fold higher with 2 μM (0.67±0.07 ng/well) of GW2974 respect to control cells (0.38±0.02 ng/well).

Effect of GW2974 on osteopontin cytosol content. To investigate the consequence of ErbB1 and ErbB2 tyrosine kinase inhibition on metastatic potential in PC-3 cell line, we measured osteopontin levels in GW2974-treated cells by ELISA test. As shown in Fig. 4, osteopontin levels were not affected by adding each individual GW2974 concentration to PC-3 cell culture medium. In fact, osteopontin mean level was not significantly different (P>0.05) in PC-3 cells treated with 0.1 μM (6.47±0.30 ng/well), 1 μM (6.48±0.32 ng/well) or 2 μM (6.58±0.28 ng/well) of GW2974 respect to vehicle-treated cells (6.44±0.38 ng/well).

Discussion

Over the last decade, data have collected to suggest that increased activity of ErbB1/ErbB2 tyrosine kinases was associated with CRPC development and biological characteristics (2,5,9,19). Lapatinib is a small molecule inhibitor of both ErbB1 and ErbB2 receptors. It causes growth arrest in different breast tumour cell types (20). In this study we determined whether GW2974, a lapatinib-like agent, had an effect on androgen-independent prostate cancer PC-3 cell line growth and NE- and metastatic-related markers cytosol content. We demonstrated that GW2974 exerts antiproliferative activity in androgen-independent prostate cancer PC-3 cell line (Fig. 1). These results agree with literature data, indicating that ErbB1/ErbB2 inhibition decreased growth in androgen-dependent and other

androgen-independent prostate cancer cell lines (7,8). Shaw and Prowse (21) found that Lapatinib treatment significantly decreased the proliferation of the androgen-independent prostate cancer cell line LNCaP C4-2B, isolated from bone metastasis of LNCaP growth in athymic mice. Our data support the key function of ErbB1/ErbB2 receptors activity in tumour cell proliferation in metastatic prostate cancer. NE cells play a pivotal role in androgen-refractory prostate tumour development and growth through the releasing of growth factors and neuropeptides that stimulate the proliferation of adjacent carcinoma cells in androgen-free environment (22). In fact prostate tumours enriched in NE cells are often more aggressive (23). Little is known about factors that promote NE cell enrichment in CRPC. We showed that GW2974 exposure of PC-3 cell line induces NE differentiation. This finding is supported by the NSE cytosol amount increase, pointed out by immunoblot analysis as well as by ELISA test (Fig. 2). Other authors have observed that ErbB1, but not ErbB2 blockade increased NSE cell content in LNCaP cell line growth in hormone-free culture conditions (10). Since GW2974 inhibits both ErbB1 and ErbB2, it should be intriguing to investigate the relative contribution of the single receptor to NE differentiation in PC-3 cell line. Conversely, Humez *et al* (9) showed that ErbB1 inhibition reduced NSE expression in DU145 androgen-independent cell line. The contrasting data obtained in DU145 cell line treated with ErbB1 inhibitor and PC-3 cell line exposed to dual ErbB1/ErbB2 inhibitor suggested that further studies are needed to clarify the ErbB2 role in NE differentiation in CRPC. Furthermore, it would be of great relevance to determine the effects of GW2974 on several other prostate cancer cell lines, particularly in DU145 cell line. Our data also indicated that chromogranin A expression was stimulated even if its content was much lower than NSE one. Previous studies were unable to detect chromogranin A in PC-3 cell line either by ELISA techniques (24) or by immunohistochemistry (17), possibly because of the lower sensitivity of used methods. Our data are supported by the recent finding of chromogranin A mRNA expression in PC-3 cell line (18).

We analyzed the effect of GW2974 on osteopontin cell content. Increased osteopontin expression is strictly linked to invasive behaviour in human breast tumour cell lines (25,26). Das *et al* (27) showed that the interaction between osteopontin and αvß3 integrin plays a key role in breast and prostate cancer metastasis. Our results indicated that ErbB1/ErbB2 inhibition had no effect on osteopontin cell content in PC-3 prostate cancer cell line. Interestingly, Zhang *et al* (28) showed that osteopontin was constitutively expressed in malignant but not in benign transformed breast cells because of constitutive activation of signal transduction molecules associated with EGF receptor. Taken together these data indicate that also in highly metastatic prostate cancer PC-3 cell line osteopontin content could not be modulated by ErbB1 nor by ErbB2 receptor inhibition. This observation reinforces the need to study the effect of ErbB1/ErbB2 activity on osteopontin expression in malignant and in benign prostate cells.

We demonstrated that in PC-3 cells ErbB1/ErbB2 blockade caused growth inhibition and NE-related marker expression increased, whereas it had no effect on osteopontin cell content. Since it was shown that somatostatin analogues such as Lanreotide in combination with other agents were able to reduce NE phenotype in patients with CRPC (29,30), it could be informative to test the response of androgen-independent prostate cancer cells to combined treatment with Lanreotide and GW2974 or Lapatinib.

Our findings may be of clinical relevance to improve novel therapies of CRPC and suggest the need to further investigate the effects of innovative ErbB1/ErbB2-targeting drugs on NE and metastatic phenotype in CRPC. This *in vitro* study may prompt an evaluation of the Lapatinib effects alone and/or in combination with NE-activity inhibitors in experimental *in vivo* models.

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