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Anti-insulin-like growth factor-I activity of a novel polysulphonated distamycin A derivative in human lung cancer cell lines

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1 The purpose of this study was to investigate the antiproliferative effect and the modulation of the mitogenic insulin-like growth factor-I (IGF-I) system by FCE 26644 and FCE 27784, two polyanionic sulphonated distamycin A derivative compounds, on two human non-small cell lung cancer (N-SCLC) cell lines.

2 For cell growth studies the colorimetric MTT and the thymidine incorporation assays were performed; the presence of IGF-I and IGF-binding proteins in conditioned media was revealed by radioimmunoassay and Western ligand blot, respectively. Variations at the IGF-I-receptor level were tested by binding studies on cell monolayers.

3 A significant concentration- and time-dependent cytostatic activity of FCE 26644 ($IC_{50} \approx 200$ μg ml^{-1} at 72 h) compared to its analogue FCE 27784 ($IC_{50} > 800$ μg ml^{-1}) was observed in both cell lines studied. The IGF-I-stimulated proliferation of the IGF-I-responsive A549 cell line was abolished by 24 h of FCE 26644 treatment whereas FCE 27784 was inactive. FCE 26644 increased (4 to 6 fold) the secretion of IGF-I-like material and reduced the IGF-I binding ($IC_{50} > 100$ μg ml^{-1}) in both A549 and Ca-Lu-1 cell lines. FCE 26644 (100 μg ml^{-1}) did not affect the K_D (≈ 0.5 nM) but reduced the B_{max} and the number of receptor sites (50%).

4 Our findings demonstrate that the ability to down-regulate the cell proliferation of N-SCLC cell lines, shown by FCE 26644, depends at least partially, on interference with the 'IGF-I mitogenic system'.

Keywords: Human lung cancer cells; distamycin A derivatives; insulin-like growth factor-I;

Introduction

Nearly 80% of bronchogenic neoplasms, the major cause of cancer-releated death in most countries (Garfinkel & Mushinski, 1994; Levi *et al*., 1994), belong to the 'non-small cell lung carcinoma' (N-SCLC) histological subtype. Despite intensive research efforts, the prognosis of N-SCLC remains extremely poor (Minna *et al*., 1989). Major advances aimed at reducing lung cancer-related mortality may be achieved through the development of novel and more effective compounds with exclusive mechanisms of action and directed toward specific tumour targets. Tumour proliferation and progression (Cross & Dexter, 1991) as well as angiogenesis (Folkman & Klagsbrun, 1987) are controlled via autocrine (Sporn & Roberts, 1985) and/or paracrine mechanisms (Aaronson, 1991), by a number of polypeptidic growth factors (GF). Among these, the growth hormone-dependent 7.6 KDa insulin-like growth factor-I (IGF-I), its 130-90 KDa heterotetrameric type-I receptor (Yarden & Ullrich, 1988) and six IGF-binding proteins (BP) (range 24–46 KDa) (Yee *et al*., 1991) constitute a system referred to as the 'IGF-I system', which has been demonstrated to contribute to the growth regulation of a variety of neoplastic diseases (Pavelic *et al*., 1986; Yee *et al*., 1991; Macaulay, 1992), including lung cancer (Minuto *et al*., 1986; Macaulay *et al*., 1988; 1990; Favoni *et al*., 1994a). A new generation of drugs, which are capable of blocking the growth factor-mediated mitogenic activity, are being investigated. The polysulphonated naphthylurea suramin (Figure 1) (Stein *et al*., 1989; Pesenti *et al*., 1992; Ravera *et al*., 1993) represents the progenitor of this new class of anticancer agents. It has been demonstrated that interference with the biochemical loop of various GFs involved in tumour proliferation and progression (Hosang, 1985; Ravera *et al*., 1993; Favoni *et al*., 1994b) as well as in the angiogenetic process (Pesenti *et al*., 1992), represents one of the main mechanisms of action of suramin. Unfortunately, the clinical use of suramin has been thwarted by its excessive toxicity and unpredictable pharmacokinetic action (Horne *et al*., 1988; Holland *et al*., 1988; Stein *et al*., 1989). Moreover, it has been shown (Pollak & Richard, 1990; Lopez-Lopez *et al*., 1992) that suramin is a highly serum-protein bound, mainly to albumin: therefore, in order to obtain the same inhibitory effect 'in vivo' as observed 'in vitro', higher concentrations of drug are required. In the search for more efficacious and less toxic drugs, Zugmaier and colleagues (Zugmaier *et al*., 1992) found that the suramin-analogue heparinoid sodium-pentosan polysulphate (Figure 1), as well as other polyanionic sugars, was able to block the paracrine stimulant effects of GFs released from tumour cells including lung cancer. More recently, a series of polyanionic naphthalene sulphonate derivatives of distamycin A (Arcamone *et al*., 1989), have been synthesized (Biasoli *et al*., 1993). These compounds have a common skeleton of four methyl-pyrrolic rings on the naphthalene ring, but vary in the number and position of the SO_3 groups. Among these, FCE 26644 (Figure 1) is active in inhibiting the coupling of heparin binding GF (HBGF-2) to its receptor *in vitro* (Ciomei *et al*., 1994). Moreover, this molecule is able to block the HBGF-2-induced neovascularisation and to inhibit solid tumour growth *in vivo* (Sola *et al*., 1995). In contrast, the compound FCE 27784 (Figure 1) has been shown to be inactive in inhibiting the hepatocyte GF-induced motogenicity of canine kidney cells (Cristiani *et al*., 1995). This paper describes the activity of FCE 26644 and FCE 27784 on two human N-SCLC cell lines. Our main pur-

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pose was to investigate the ability of these molecules to interfere with the potent 'IGF-I mitogenic system' implicated in the regulation of proliferation of the cell lines under study.

Methods

Cell lines

Human N-SCLC A549 and Ca-Lu-1 cell lines belonging to the adenocarcinoma and squamous carcinoma histological subtype, respectively, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained in Dulbecco's modified Eagles's medium (DMEM) supplemented with 5% heat-inactivated foetal calf serum (HI-FCS), glutamine (2 mM), antibiotics (0.02 u ml^{-1} penicillin and 0.02 μg ml^{-1} streptomycin) and non-essential amino acids (1%). For experiments performed in serum-free medium (SFM), DMEM containing glutamine, transferrin (2 mg ml^{-1}), 4-(2-hydroxy-ethyl)-1-piperazine ethane sulphonic acid buffer (HEPES, 20 mM) and trace elements (1%) was used.

Cell proliferation studies

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay This was performed as described previously (Carmichael *et al*., 1987). Exponentially growing cells, harvested by trypsinization and resuspended in DMEM + 5% HI-FCS, were plated in quadruplicate in 96-well microtiter plates at a density of 3×10^3 cells/well in 200 μl of serum-containing medium. The initial quantity of seeded cells was selected in order that untreated cultures remained in the exponential growth phase until the end of the experiments. After overnight incubation at 37°C, medium was removed and replaced with fresh medium in the presence or absence of FCE 26644 or FCE 27784 (from 50 to 400 μg ml^{-1}). After 48 and 72 h, 50 μl of MTT (2 mg ml^{-1}) were added to each well and the cells were incubated for 4 h. Plates were then treated as described by de Cupis *et al*. (1995).

For cell growth recovery evaluation 24 h after plating, cells were exposed to FCE 26644 (50–400 μg ml^{-1}) for 48 h. After this time, medium was removed and replaced with fresh medium in the presence or absence of the drug. Cells were incubated for two days before the colorimetric assay.

DNA synthesis evaluation Cell growth inhibition by FCE 26644 or by FCE 27784, used either alone or in combination with exogenous IGF-I, was evaluated by the [methyl ^{3}H]-thymidine ([^{3}H]-dThd) incorporation assay as previously decribed (Favoni *et al*., 1994b). Briefly, a viable single cell suspension was plated in triplicate in 35 mm diameter dishes at a density of 10^5 cells/well in 2 ml of DMEM + 5% HI-FCS. For the concentration-response studies, performed either in serum-deprived or in serum-containing medium, cells were plated and after 24 h they were then exposed to the drugs at concentrations ranging from 25 to 800 μg ml^{-1} for 24 and 48 h. In order to verify the possible modulation caused by serum proteins on drug-induced cell growth inhibition and to test the behaviour of each individual drug, cells were incubated in increasing concentrations of FCS (1–20%) in the presence of 200 μg ml^{-1} of FCE 26644 or suramin. After 24 and 48 h of

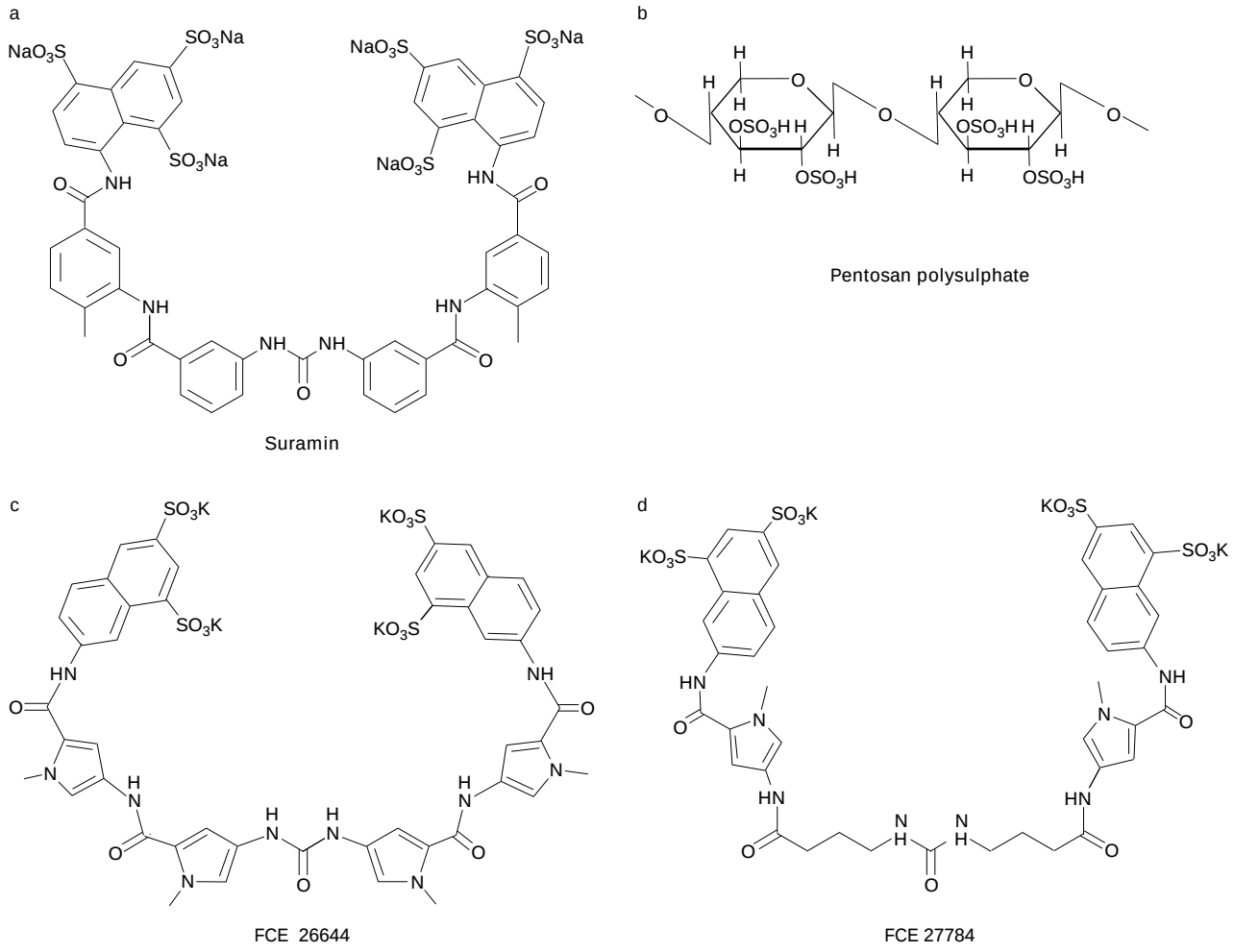

Figure 1 Chemical structures of polysulphonated naphthylamine suramin (a), building blocks of pentosan polysulphate (b) and polysulphonated distamycin A derivatives FCE 26644 (c) and FCE 27784 (d) cited in this study.

treatment, DNA synthesis was evaluated. To investigate the drug-related effect on IGF-I-induced cell proliferation, the IGF-I-sensitive A549 adenocarcinoma cell line was exposed to 10 nM IGF-I and 100 or 200 μg ml^{-1} of FCE 26644 or FCE 27784 (in SFM), alone or in combination for one or two days before measurement of tritiated thymidine incorporation into DNA.

Conditioned media preparation

Conditioned media (CM), collected from cells grown either in SFM or in 10% serum-containing medium for 48 h in the presence or absence of 100 μg ml^{-1} of drug and the addition of protease inhibitors (0.1 M phenyl-methyl-sulphonyl fluoride (PMSF), 50 μg leupeptin and 0.01 mM pepstatin-A 100 ml^{-1} of CM), were prepared and appropriately concentrated as described by Favoni *et al*. (1994a).

Evaluation of cell-secreted IGF-I-like material by radioimmunoassay

Quantitation of immunoreactive IGF-I-like material was performed on 30× concentrated CM by competitive radioimmunoassay (RIA) in the presence of Na-heparin to promote the dissociation of the IGF-I/BP complex (Favoni *et al*., 1995). Increasing concentrations of human recombinant IGF-I (hr-IGF-I) for the standard curve (0.025 to 400 ng ml^{-1}) as well as unknowns were diluted in 300 μl of RIA buffer (0.03 M $NaHPO_4$, 0.25% w/v RIA grade bovine serum albumin (BSA), 0.02% w/v Na-azide and 1 u ml^{-1} Na-heparin). These were co-incubated for 96 h at 4°C with 50 μl of an anti-IGF-I rabbit polyclonal antibody (UBK 487) used at a final dilution of 1 : 18,000. At the end of this incubation, 100 μl of [^{125}I]-IGF-I ($\approx$20,000 c.p.m.) were added to each sample and incubated overnight at 4°C. Antigen-antibody complex was precipitated by the addition of 100 μl/polypropilene tube of 1 : 10 Pansorbin-A cells and after centrifugation at 13,000 r.p.m. for 10 min at 4°C, the radioactivity in the pellet was evaluated in a Beckman 5500 B γ-counter.

Detection of IGF-BPs secreted in conditioned media

The detection of the presence of IGF-BPs secreted in CM, either with or without FCE 26644 treatment (100 μg ml^{-1}), was performed by Western ligand blot (WLB). Total protein content in the CM was measured by the Bradford dye-binding method (Bio-Rad protein assay, Munich-Germany) in order that a volume corresponding to 10 μg of total protein was loaded for each sample. Proteins were resolved in a 15% SDS–PAGE as described previously (Favoni *et al*., 1994a) and transferred to a nitrocellulose membrane. After hybridization with radiolabelled IGF-I, the blots were exposed to X-ray film for five days at −80°C. Drug interference with the secretion of IGF-BP was evaluated by visual observation of the autoradiography.

Binding studies

The influence of FCE 26644- or FCE 27784-treatment on IGF-I binding to its cell surface receptor was evaluated by radio receptor assay (RRA) by use of the following standard protocol. Cells were plated at a density of 2×10^5 (A549) and 2.5×10^5 (Ca-Lu-1) in 1 ml serum-containing DMEM in 24-well multiwell plates. After 24 h monolayers were washed and incubated for 1 h at 37°C in binding buffer (DMEM supplemented with 0.1% BSA, 40 mM HEPES. Cells were then co-incubated with agitation, for two hours at 4°C with iodinated IGF-I ($\approx$25–30,000 c.p.m.) together with serial dilutions of unlabelled growth factor (from 0.01 to 2.63 pmol) and 100 μg ml^{-1} of FCE 26644 (FCE 27784) or with radiolabelled IGF-I and increasing concentrations of drugs (from 25 to 800 μg ml^{-1}) in 400 μl binding buffer. At the end of this period, cell monolayers were washed and solubilized as previously described (Favoni *et al*., 1994b): radioactivity in the cell lysate was detected in a Beckman 5500 B γ-counter. In order to ascertain whether FCE 26644 modulates the GF binding by interfering at the IGF-I receptor rather than at the ligand level, RRAs were performed with variations in the sequence of addition of reagents. Initial studies constituted incubating cell monolayers with the sulphonate distamycin A derivative (from 25 to 800 μg ml^{-1}) for 2 h at 4°C. After this period the cells (rinsed or unrinsed) were incubated, with agitation, with iodinated IGF-I ($\approx$25–30,000 c.p.m.) for 2 h at 4°C. In a second set of assays, labelled IGF-I and a serial dilution of the drug were pre-incubated (2 h, 4°C) together before their administration to cells in the presence of unlabelled IGF-I (0.01, 0.16 and 21 pmol) for two hours. Finally, in a third set of experiments, iodinated and unlabelled growth factor were administered to the cell monolayer at the same time and temperature conditions as decribed above. Serial dilutions of drug were then added to each well for 2 h. At the end of each set of assays, cells were treated as described above.

Drugs and chemicals

FCE 26644 (batch ≠94B011, $C_{45}H_{36}N_{10}O_{17}S_4K_4$; MW = 1273.5) and FCE 27784 (batch ≠PA10201/24, $C_{41}H_{38}N_8O_{17}S_4K_4$; MW = 1199.24) were obtained from Pharmacia (Nerviano, Mi, Italy). Stock solutions of 10 mg ml^{-1} were prepared in double-distilled water and were stored at −20°C. Suramin (MW = 1429.2), diluted and stored as for FCEs, was a gift of Bayer AG (Leverkusen, Germany). Lyophilised pure hr-IGF-I (PeproTech Inc., Rocky Hill, N.J., U.S.A.) was reconstituted in 0.1 N acetic acid at a concentration of 10 μg 100 μl^{-1} and stored at −20°C. MTT, BSA RIA-grade, PMSF, leupeptin and pepstatin-A were obtained from Sigma (St. Louis, MO, U.S.A.). The anti-IGF-I rabbit polyclonal antibody (UBK 487) was acquired from the National Hormone and Pituitary Program distributed by the Hormone Distribution Program of the National Institute for Diabetes, Digestive and Kidney Diseases (Bethesda, MD, U.S.A.). Pansorbin-A cells (*Staphylococcus aureus*) were from Calbiochem (La Jolla, CA, U.S.A.). Tissue-culture medium and HEPES were obtained from ICN (Milan, Italy). Mycoplasma-virus screened foetal calf serum as well as trace elements were acquired from Gibco (Milan, Italy). [^{3}H]-dThd (TRA120, specific activity, s.a. = 5 Ci mmol^{-1}, 185 GBq mmol^{-1}) was reconstituted in phosphate buffered saline, stored in aliquots of 160 μCi ml^{-1} at 4°C. Radiolabelled [^{125}I]-IGF-I (IM172, s.a. $\approx$2000 Ci mmol^{-1}, 74 TBq mmol^{-1}) reconstituted with 0.1 N acetic acid was stored in aliquots of 2 μCi 20 μl^{-1} at −20°C (Amersham Int. U.K.). Tissue-culture material was purchased from Becton-Dickinson (Parsipanny, NJ, U.S.A.) and Greiner (Nutrigen, Germany).

Statistical analysis

Results of cell growth studies (colorimetric MTT assay and DNA synthesis evaluation) as well as radioimmuno (RIA) and binding (RRA) assays are expressed as a mean of percentages ± s.e. of four experiments performed in quadruplicate (MTT), triplicate ([^{3}H]-dThd) and duplicate (RIA and RRA). Analysis of variance between treated and untreated groups was evaluated by the non-parametric Wilcoxon-test.

Results

Effect of FCE 26644- and FCE 27784-treatment on cellular proliferation

The MTT assay (Figure 2) revealed a significant ($P = 0.001$) concentration- and time-dependent cell growth inhibition which was more evident after 72 h of FCE 26644 exposure, in both A549 and Ca-Lu-1 N-SCLC cell lines ($IC_{50} > 400$ μg ml^{-1} and $\approx$200 μg ml^{-1} at 48 and 72 h). At the shortest time

of exposure (48 h), FCE 26644 concentrations below 100 μg ml^{-1} provoked an increase in cellular proliferation which was more evident in the A549 cell line. In contrast, FCE 27784 did not effect the cell growth ($IC_{50} > 800$ μg ml^{-1} at 48 and 72 h) of either cell line (data not shown).

The [^{3}H]-dThd incorporation assay which in contrast to the MTT experiments was performed in serum-depleted medium, indicated a concentration- (25 to 800 μg ml^{-1}) and time- (24 and 48 h) dependent decrease of DNA synthesis in the Ca-Lu-1 squamous cell line after FCE 26644 exposure ($IC_{50} > 100$ μg ml^{-1} at 24 h and equal to 100 μg ml^{-1} at 48 h). However, the A549 cell line behaved differently: growth inhibition which was not concentration-dependent reached a maximum of 55% at 25 μg ml^{-1} and did not change with increasing concentrations of FCE 26644 (data not shown). Under serum-free conditions FCE 27784 was unable to down-regulate the A549 and Ca-Lu-1 cell growth (data not shown). [^{3}H]-dThd incorporation assays performed at increasing percentages of serum (1 to 10%) revealed an attenuation of the inhibitory action of the drugs. This effect, which was more evident at 48 h and was FCS concentration-dependent only for FCE 26644, is probably due to drug capture by serum proteins. At equal serum concentrations the inhibitory activity of suramin was ($P = 0.002$) affected more ($\approx$two fold; ranging from 3.2 to 1.3 fold) than FCE 26644. No significant differences between the two drugs were observed at the highest FCS concentration (data not shown).

In order to verify that FCE 26644 was inducing a cytostatic rather than cytotoxic effect, a cell growth recovery assay was carried out by MTT assay. Cells exposed for 48 h to renewed medium after 2 days of drug exposure (50 to 400 μg ml^{-1}) had almost completely recovered from the drug-induced cell growth inhibition in both N-SCLC cell lines (data not shown).

We have recently demonstrated (Favoni *et al*., 1994a) the presence and the functionality of the 'IGF-I mitogenic system' in a pool of N-SCLC cell lines, including those used in this study. We have shown that the IGF-I-stimulated cell proliferation increased (47%; $P = 0.001$) only in the A549 adenocarcinoma histological subtype. Here, we investigated the effect on DNA synthesis of the co-administration of 100 or 200 μg ml^{-1} of FCE 26644 and FCE 27784 with 10 nM of IGF-I (24 and 48 h) in serum-depleted medium on the A549 cell line. After one day of exposure, FCE 26644 abolished the mitogenic effect of exogenous IGF-I at both concentrations used (Figure 3), whereas FCE 27784 was inactive (data not shown). Since FCE 27784 was inactive in reducing cellular proliferation and in reversing the biological effects of exogenous IGF-I, the evaluations of a possible 'IGF-I system'-inhibitor were restricted to FCE 26644.

Modulation of the secretion of IGF-I-like material in conditioned media (CM)

To investigate whether exposure to FCE 26644 (100 μg ml^{-1}, 48 h) is able to interfere with the production of the growth factor, we performed a radioimmunoassay in the presence of heparin, on 30 $\times$ concentrated (c)CM obtained from cells grown either in serum-depleted or in 10% serum-containing medium. FCE 26644 provoked an increase of immunoreactive IGF-I-like material in relation to the total amount of protein, in both A549 (>4 fold) and Ca-Lu-1 (>6 fold) cell lines cultured in SFM (Table 1). By contrast, the cCM collected from cells grown in complete medium, showed a consistent drug-induced decrease ($\approx$1.4 fold in A549 and $\approx$2.7 fold in Ca-Lu-1 N-SCLC cell lines) in growth factor secretion (data not shown).

Effect of drug treatment on expression of IGF-binding proteins

Figure 4 shows results of a Western ligand blot (WLB) performed on 30 $\times$ cCM of the CaLu-1 squamous carcinoma cell line. Visual evaluation of the autoradiography clearly indicates that FCE 26644 exposure (100 μg ml^{-1} for 48 h) caused a

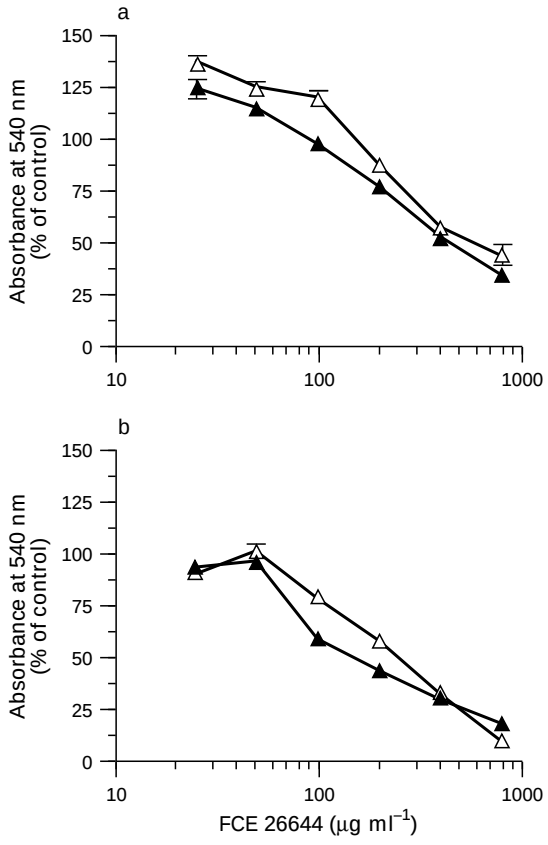

Figure 2 Effect of FCE 26644, at concentrations from 25 to 800 μg ml^{-1}, on cell proliferation of A549 (△) and Ca-Lu-1 (▲) N-SCLC cell lines. Cells were plated in 96-well microplates (3×10^3 cells/well in 200 μl of medium) in DMEM supplemented with 5% FCS and exposed the day after plating, to the drug for (a) 48 and (b) 72 h. At the end of each time point cells were exposed to 50 μl of 2 mg ml^{-1} MTT stock solution for 4 h at 37°C and then treated as described in Methods section. Data, expressed as % of the control, represent the absorbance measured at 540 nm in a spectrophotometer.

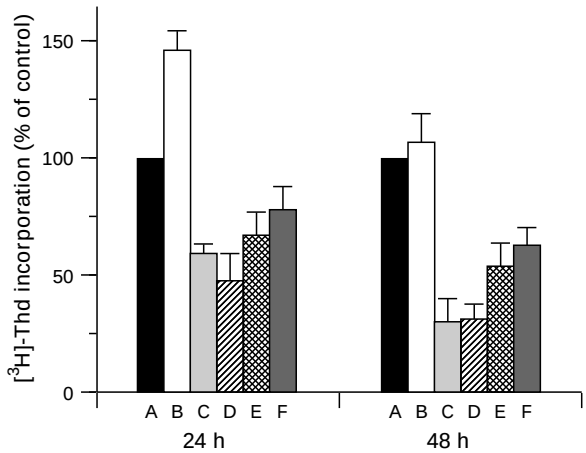

Figure 3 Histograms showing the effect of 24 and 48 h of 100 (columns C) and 200 μg ml^{-1} (columns D) FCE 26644, alone or in combination with IGF-I (columns B, 10 nM IGF-I; columns E, 100 μg ml^{-1} FCE 26644 + 10 nM IGF-I; columns F, 200 μg ml^{-1} FCE 26644 + 10 nM IGF-I), on A549 cell growth, evaluated by thymidine incorporation assay. Values, expressed as % of control (columns A), are the mean of four independent experiments, performed in triplicate, $\pm$s.e. (ranging from 4 to 11%).

Table 1 Modulation of immunoreactive IGF-I-like material secreted in CM of N-SCLC cell lines induced by FCE 26644

Cell lines	*Total protein*[a] (μg ml^{-1})	*Immunoreactive IGF-I material*[b] (ng ml^{-1})	(ng μg^{-1} total protein)	Increase (fold)
A549				
Untreated	11.0	4.2	0.39	
FCE 26644[c]	4.0	6.7	1.67	>4
Ca-Lu-1				
Untreated	10.1	18.0	1.78	
FCE 26644[c]	3.3	39.0	11.80	>6

[a]Referred to 1x conditioned media (CM). [b]RIA was performed in buffer supplemented with heparin (1 u ml^{-1}). [c]Concentration 100 μg ml^{-1}, 48 h.

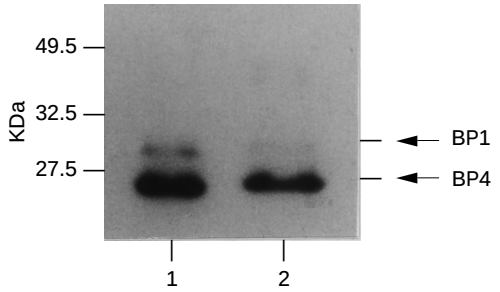

Figure 4 Ligand blot of untreated (lane 1) and 100 μg ml^{-1} FCE 26644-treated (lane 2) 30 × concentrated conditioned medium (cCM) from CaLu-1 N-SCLC cell line. The molecular weight standards are annotated in the left hand panel whereas on the right hand side the arrows indicate the molecular weight of IGF-BP4 and IGF-BP1.

decrease in IGF-BP4 (24 KDa) and IGF-BP1 (30 KDa) expression.

Binding studies

Co-incubation (2 h at 4°C) of [^{125}I]-IGF-I with FCE 26644 (ranging from 25 to 800 μg ml^{-1}) resulted in a concentration-dependent reduction of the growth factor binding in both A549 and Ca-Lu-1 cell lines (IC_{50} > 100 μg ml^{-1}). At 25 μg ml^{-1} the Ca-Lu-1 squamous carcinoma cell line was slightly more sensitive than the A549 cell line to inhibition of IGF-I binding (Figure 5). Scatchard analysis of the data performed with a Binding Analysis Computer Programme (McPherson, 1985), revealed that 100 μg ml^{-1} FCE 26644 caused a 45% average decrease in B_{max} values as well as in the number of receptor sites (50%) in both cell lines. No variation was observed in the affinity constant (K_D) values (Table 2). Pretreatment of cells with FCE 26644 for 2 h did not prevent the binding of subsequently administered growth factor to its cell surface receptor. If cells were treated (2 h at 4°C) with medium containing [^{125}I]-IGF-I and previously co-incubated for 2 h at 4°C giving them the possibility to bind together, no labelled IGF-I remained available (in a concentration-dependent manner) to compete for the receptor-binding. Moreover, we noted that if already pre-constituted, FCE 26644 even at 800 μg ml^{-1} was unable to dissociate the growth factor/growth factor receptor complex (Figure 5).

Discussion

The aim of the present study was to investigate the anti-proliferative potential of FCE 26644 and FCE 27784 on two human non-small cell lung cancer cell lines, with particular emphasis on their suitability as inhibitors of IGF-I-controlled

Table 2 Effect of FCE 26644 100 μg ml^{-1} on equilibrium dissociation constant, maximum binding capacity and number of sites/cell on N-SCLC cell lines

Cell lines	*Untreated* K_D (nM)	B_{max} (pM)	*Sites* $\times 10^4$/cell	*FCE 26644 (100 μg ml^{-1})* K_D (nM)	B_{max} (pM)	*Sites* $\times 10^4$/cell
A549	0.53	80	18	0.47	39	9
Ca-Lu-1	0.53	27	6	0.53	16	3

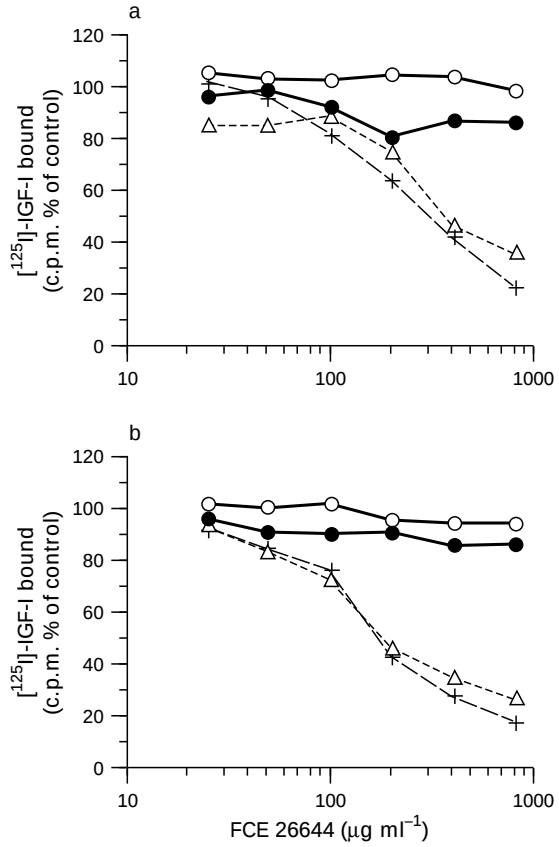

Figure 5 The effect on [^{125}I]-IGF-I binding induced by FCE 26644 (25 to 800 μg ml^{-1}) as evaluated by either the standard radioreceptor assay protocol (+) (see in Methods section) or its modifications, as follows: drug administered to (a) A549 and (b) Ca-Lu-1 cell lines 2 h before the addition of the labelled growth factor (○); drug administered to cells 2 h after the addition of the labelled growth factor (●); drug and labelled growth factor pre-incubated together for 2 h before their addition to cells (△). At the end of each set of assays, cell monolayers were washed and lysed: the [^{125}I]-IGF-I bound to cells was then measured in a Beckman γ-counter. Results, expressed as % of control, are the mean of four experiments performed in duplicate (s.e. were very low, <4%, therefore difficult to represent on the graph).

proliferation. Growth of both A549 and Ca-Lu-1 human N-SCLC cell lines was significantly inhibited in a cytostatic and concentration-/time-dependent manner by FCE 26644 exposure, whereas FCE 27784 was inactive. These findings are in agreement with the results obtained by Cristiani and colleagues (Cristiani *et al*., 1995) for inhibition of hepatocyte growth factor activity. We also found that the Ca-Lu-1 squamous N-SCLC cell line was more sensitive to FCE 26644 exposure than the adenocarcinoma A549 cell line. This behaviour is in agreement with that found in our previous study showing the stronger down-regulation of cellular proliferation in response to suramin exhibited by Ca-Lu-1 as compared to the A549 cell line (Favoni *et al*., 1994b). Experiments carried out in serum-free medium confirmed the higher sensitivity of the squamous

carcinoma cell line to FCE 26644 exposure. Increased expression of various autocrine and paracrine growth factors (Minuto *et al*., 1986), including IGF-I (Favoni *et al*., 1994a) in non-small cell lung cancer cell lines, has been demonstrated. Hence, we investigated the ability of FCE 26644 to interfere with the IGF-I-induced mitogenicity. The increased cell growth promoted by exogenous IGF-I on the A549 adenocarcinoma cell line was dramatically reduced by simultaneous exposure to FCE 26644. By contrast, the cellular proliferation of the squamous Ca-Lu-1 cell line was not affected by the growth factor treatment. We hypothesize that IGF-I or a closely related immunoreactive species, whose production by this cell line was revealed by both radioimmunoassay and Northern blot analysis (Favoni *et al*., 1994a), operates as an autocrine growth factor. Thus, the inhibitory activity of FCE 26644 on Ca-Lu-1 may be related to the interference with an autocrine growth factor/growth factor-receptor loop. The secretion of IGF-I-like material in conditioned media, which was strongly increased by the drug exposure, probably represents an attempt by the cell to survive in the poor growth-maintenance environment constituted by the minimum-essential medium. This supposition appears to be substantiated by the observation that when cells were cultured in serum-containing medium, growth factor secretion was decreased by the drug exposure. On consideration of the higher Ca-Lu-1 vs. A549 FCE 26644-responsiveness together with the greater secretion of the probably autocrine action of IGF-I which follow the drug exposure, Ca-Lu-1 conditioned medium was chosen for the analysis of the expression of IGF-BPs. Therefore, a significantly higher drug-related inhibition of cell growth in the Ca-Lu-1 squamous carcinoma cell line could be partially related to the observed decreased production of IGF-BPs. Binding studies were performed on the basis of previous observations indicating that FCE 26644, as well as other distamycin A derivatives (Ciomei *et al*., 1994; Sola *et al*., 1995), suramin (Ravera *et al*., 1993; Favoni *et al*., 1994b) and its pentosan polysulphated analogues (Zugmaier *et al*., 1992), showed a growth-inhibitory effect by complexing various growth factors. An absence of variations in K_D values and in the growth factor binding induced by drug pretreatment, as well as a decrease of ligand availability, following the simultaneous incubation of drug and iodinated IGF-I in the absence of cells, was observed. These findings suggest that FCE 26644 does not compete directly at the receptor level, but interferes with the IGF-I/IGF-receptor interaction by capturing the growth factor itself and eventually preventing its binding. Furthermore, the finding that the drug was unable to dissociate a pre-formed growth factor/growth factor receptor complex substantiates the supposition that the polyanionic drugs bind preferentially to the polypeptidic growth factors as opposed to their related receptors. This mechanism of action was also suggested by Cristiani and co-workers (Cristiani *et al*., 1995) who observed that treatment with FCE 26644 inhibited neither HGF-independent kinase activity of c-Met, nor epidermal growth factor-induced receptor activation. Their results lead them to conclude that the drug selectively inhibits HGF action by interfering with the ligand/receptor loop in the c-Met-HGF system. The availability of new polysulphonated drugs like FCE 26644, endowed with lower toxicity and easier use, could constitute important developments in the future of tumour chemotherapy. The capability to hinder the neoplastic proliferation by switching-off the network of cell-produced growth factor interconnected pathways, provides a stimulating model for planning new chemotherapeutic strategies. In light of this theory, the entire 'IGF-I system', particularly the potent mitogenic ligand, could be considered to be among the most important new targets for more selective antimitogenic drugs controlling the growth of N-SCLC, thus the new compound FCE 26644 could accomplish this role. In conclusion, FCE 26644 is an interesting agent with pre-clinical antitumour activity in N-SCLC. Given its more favourable toxicity profile, as observed in the preliminary study on different solid murine tumour models (Sola *et al*., 1995) as compared to equimolar doses of its parental compound suramin, FCE 26644 deserves clinical development and testing in N-SCLC and other solid tumours.

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