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(54) Title: IMPROVEMENTS IN OR RELATING TO AGENTS FOR THE TREATMENT OF CARDIOVASCULAR DYSFUNCTION

(57) Abstract: Use of a EGFR tyrosine kinase inhibitor in the manufacture of a medicament for the therapeutic or prophylactic treatment of cardiovascular dysfunction, especially cardiovascular dysfunction which is non-proliferative in origin and/or characterised by abnormal vascular reactivity. Suitable inhibitors include derivatives of quinoline, of quinazoline, of isoquinoline and of indole derivatives. Inhibitors are preferably specific for EGFR.



WO 2004/069232 A2

Improvements in or relating to agents for the treatment
of cardiovascular dysfunction

The present invention relates to novel applications
5 of substances which inhibit certain tyrosine kinase
activity.

Tyrosine kinases are known to be critical in the
proliferation pathway which, on failure of the normal
control mechanism, gives rise to uncontrolled
10 proliferation of cancer cells. The epidermal growth
factor receptor ("EGFR") is known to regulate a number of
cellular processes through tyrosine phosphorylation.
EGFR is a catalytic receptor protein. It passes through
the cell-surface membrane once and has a catalytic domain
15 exposed on the cytoplasmic side of the membrane and a
ligand-binding domain exposed on the extra-cellular side
of the membrane. The tyrosine kinase activity of EGFR
activity is activated by ligand binding to the receptor.
It is therefore possible to inhibit the tyrosine kinase
20 activity of EGFR at various different target sites
present in EGFR. For example, an inhibitor of EGFR
tyrosine kinase might compete with ligand for EGFR
binding or might interfere directly with the catalytic
site of EGFR. Alternatively or additionally, EGFR
25 tyrosine kinase activity may be inhibited by preventing
formation of the receptor protein altogether such as is
possible with gene silencing agents, for example SiRNAs
and antisense oligonucleotides. The term "EGFR tyrosine
kinase inhibitor" includes all inhibitors of the tyrosine
30 kinase activity of EGFR regardless of their mode of
action.

A number of potential therapeutic agents which are inhibitors of EGFR tyrosine kinase have been previously identified as having anti-proliferative activity (see, for example, WO 96/33980 and WO 96/30347), and have
5 accordingly been proposed for use in anti-cancer therapy.

Enhanced levels and/or activity of EGFR have been observed in the gastric mucosa of diabetic subjects, where it is thought to lead to increased sensitivity of the gastric mucosa to injury (Khan et al, Proc. Soc. Exp.
10 Biol. Med. (1999) 221 105 - 110), and also in the kidney of diabetic animals, where excessive local synthesis and release of EGF may contribute to the interstitial fibrosis in diabetic kidney tubules (Ziyadeh & Goldfarb, Kidney Int. 39, 464 - 75).

15 G-protein coupled receptor signalling, including transactivation of receptor tyrosine kinases ("RTKs") has been implicated in vascular pathology. The role of individual RTKs in the development of cardiovascular dysfunction, and especially in diabetes-
20 induced cardiovascular dysfunction, is unknown.

Signalling via RTKs has an influence on intracellular calcium levels, and has been implicated in the development of pathologies in hypertension and diabetes (Patel et al, Br. J. Ophthalmol. 78, 714 - 718
25 (1994); Khan et al; Exp. Biol. Med. 221, 105 - 110 (1999); Muthalif et al. Proc. Natl. Acad. Sci. U.S.A. 95, 12701 - 12706 (1998); Muthalif et al. Hypertension 35, 457 - 463 (2000; Muthalif et al. Hypertension 36, 604 - 609 (2000); Muthalif et al. Hypertension 39, 707 - 709;
30 Kassab et al, Vasc. Med. 6, 249-255 (2001); Nakajima et al. Invest. Ophthalmol. Vis. Sci. 42, 2110 - 2114 (2001)). In diabetic rats fed on a diet containing

genistein, a broad spectrum inhibitor of tyrosine kinases, normalization of retinal vascular leakage has been observed (Nakajima et al, vide supra). The precise mechanism of that normalization is, however, unknown.

5 It has been suggested that, in vascular smooth muscle cells and experimental models of hypertension, activation of the cPLA₂/cytochrome P-450 (CYP-450)/Ras/Raf/MAPK pathway by various agents (angiotensin II, norepinephrine or epidermal growth factor) leads,
10 through elevation of intracellular calcium levels, to the development of hypertension and related end-organ pathologies.

 A significance of the contribution of EGFR tyrosine kinase signalling in the development of cardiovascular
15 dysfunction, and in particular diabetes-induced cardiovascular dysfunction, has however not been recognised to date.

 The present invention provides use of a EGFR tyrosine kinase inhibitor in the manufacture of a
20 medicament for use in the therapeutic or prophylactic treatment of cardiovascular dysfunction.

 The present inventor has found that, surprisingly, inhibition of EGFR tyrosine kinase activity can give rise to normalization of vascular function in cardiovascular
25 dysfunctional subjects especially those with diabetes-induced cardiovascular dysfunction.

 In particular, the inventor has found that EGFR tyrosine kinase is involved in signalling pathways leading to abnormal vascular reactivity, in particular in
30 diabetes.

 The inventor has discovered that EGFR signalling is important in mediating diabetic vascular dysfunction, and

it is believed that that mediation occurs via modulation of calcium homeostasis, which leads to altered responsiveness to vasoconstrictors and/or vasodilators. Whilst not wishing to be bound by theory, it is thought
5 that the modulation of calcium homeostasis includes enhanced calcium sensitivity and/or increased calcium entry through metabolism of arachidonic acid via Cytochrome P-450 to 20-HETE, a potent vasoconstrictor which, possibly, then activates the EGFR/shc/Grb2/sos/Ras
10 pathway. The inventor's evidence below that inhibition of EGFR tyrosine kinase activity normalizes vascular function in diabetics supports that view.

Accordingly, one embodiment of the invention provides use of an EGFR tyrosine kinase inhibitor in the
15 manufacture of a medicament for the therapeutic or prophylactic treatment of cardiovascular dysfunction wherein the cardiovascular dysfunction is non-proliferative in origin. The term "non-proliferative in origin" excludes disorders wherein a significant part of
20 the pathology is caused by cell proliferation. The term therefore excludes cancer. It also excludes disease such as restenosis wherein the pathology is caused by excessive non-malignant cellular proliferation. The term "non-proliferative in origin" does not exclude disorders
25 which may yet progress to acquire a proliferative aspect but which have not yet done so to a significant extent.

Preferably, the cardiovascular dysfunction is characterized by abnormal vascular reactivity. Disorders known to be associated with abnormal vascular reactivity
30 include retinopathy, nephropathy and male erectile dysfunction. The aetiology of these disorders may be associated with other disorders, especially diabetes and

hypertension, and for that reason, the present invention is particularly applicable to the treatment of cardiovascular dysfunction in subjects who are diabetic or hypertensive. However, cardiovascular dysfunction
5 characterized by abnormal vascular reactivity is not solely associated with diabetes nor hypertension and the present invention is also applicable to the treatment of retinopathy, nephropathy and male erectile dysfunction having non-diabetic and/or non-hypertensive aetiology.

10 There is evidence that abnormal vascular reactivity may be a contributory factor in the development of stroke. Accordingly, in one embodiment the present invention provides use of an EGFR tyrosine kinase inhibitor in the manufacture of a medicament for use in
15 the therapeutic or prophylactic treatment of stroke.

 The inhibitor will be administered in a therapeutically or prophylactically effective amount. The amount of the inhibitor to be administered will depend on the activity of the inhibitor, on the form of
20 administration and in therapeutic use on the severity of the affliction. In general, effective dosages will be from about 0.001 to 100 mg/kg/day, for example, from 1 to 50 mg/kg/day. Where, as is preferred, administration is in the form of oral dosage units, for example, tablets or
25 capsules, each dosage unit will advantageously contain from 50 to 500 mg, and preferably from 100 to 400 mg, for example, 250 mg of the active substance.

 The active substance may be presented for administration by any suitable route, for example,
30 orally, by injection, by inhalation or transdermally. Suitable dosage forms include, for example, liquids, solutions or suspensions in orally administrable form, or

in injectable form for injection intravenously, subcutaneously or otherwise; tablets or capsules for oral ingestion; gels, creams or ointments for transdermal administration; and powders, solutions or suspensions for pulmonary, intranasal or buccal administration.

Preferred dosage forms are oral dosage forms, especially tablets or capsules for oral use, which may if desired be provided with a coating or other means for providing controlled or delayed release of the active substance.

The medicaments of the invention will normally comprise the reactive substance in combination with one or more additional ingredients selected from carriers, excipients and adjuvants.

The tyrosine kinase inhibitor may be a bicyclic heteroaromatic derivative with at least one nitrogen atom in the heterocyclic ring system, especially a heteroaromatic derivative selected from derivatives of quinoline, of quinazoline, of isoquinoline or of indole.

In one embodiment of the invention, the tyrosine kinase inhibitor is a quinazoline derivative, especially an anilino quinazoline or a derivative thereof.

In a further embodiment the tyrosine kinase inhibitor may be a quinoline derivative, especially an anilino quinoline, or a derivative thereof.

Advantageously, the quinazoline or quinoline derivatives is substituted at the 6- and/or 7-position by an R^1 -O-group or an R^2 -N-group in which R^1 and R^2 can be the same or different and each can be an aliphatic group which may be saturated or unsaturated and preferably contains from 1 to 6 carbon atoms in the aliphatic chain, the aliphatic chain optionally being interrupted by one or more heteroatoms, especially oxygen and/or being

interrupted or terminated by one or more functional groups, for example a -CO-group, and/or being interrupted or terminated by one or more cyclic group, for example a morpholine group and/or an aromatic or heteroaromatic group, and/or being interrupted or terminated by an amino group.

Advantageously, the anilino ring of the quinoline or quinazoline derivatives is halogenated, especially at the 3-position, for example chlorinated at the 3-position, and/or optionally substituted, for example halogenated, at the 4-position, especially fluorinated at the 4-position.

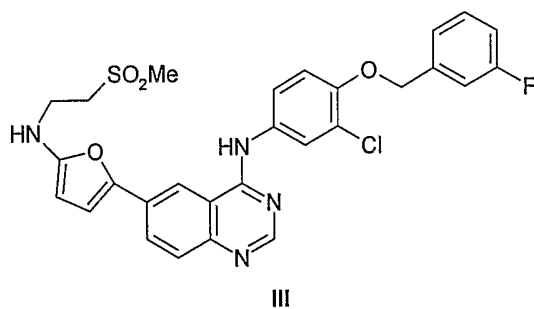
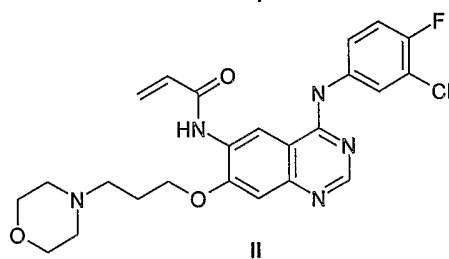
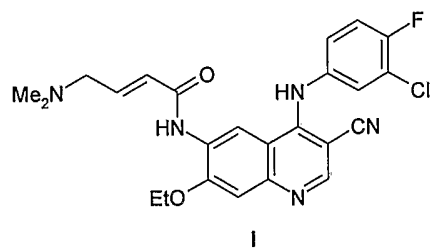
One class of tyrosine kinase inhibitors which has been found to have useful anti-tumour activity is quinazoline derivatives having an anilino substituent at the 4-position, a dialkylaminoalkoxy substituent at the 7-position and an alkoxy substituent at the 7-position, as disclosed in International Patent Application No. WO 96/33980 (Astra Zeneca). The contents of that document, and in particular the general formula I thereof, are incorporated herein by reference. One compound according to WO 96/33980 is currently undergoing clinical trials for anti-cancer activity under the name IRESSA (registered trade mark - Astra Zeneca UK Limited), formula V herein. WO 96/33980 states that it is expected that the quinazoline derivatives will, in addition to treatment of cancers, be useful in the treatment of other disorders of cellular growth in which aberrant cell signalling by way of receptor tyrosine kinase enzymes or non-receptor tyrosine kinase enzymes, including as yet unidentified tyrosine kinase enzymes are involved, and suggests that such disorders include inter alia, vascular

restenosis. It also states that it is expected that inhibitors of EGF type receptor tyrosine kinases will be useful in the treatment of non-malignant diseases of excessive cellular proliferation such, as inter alia,
5 atherosclerosis and restenosis. There is no data, however, in WO 96/33980 supporting those hypotheses.

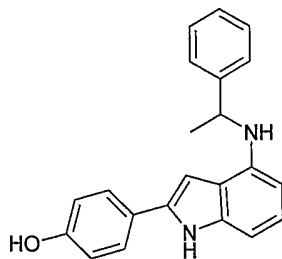
Another class of anilino quinazoline derivatives having anti-tumour activity is described in WO 96/30347 (Pfizer), represented by the drug TARCEVA (trade mark),
10 formula VI herein, which has also been undergoing trials as an anti-cancer therapy. The disclosure of WO 96/30347, and in particular formula I thereof, is incorporated herein by reference.

A number of other EGFR tyrosine kinase inhibitors
15 are currently in development or undergoing trials. At present their development is being driven by their putative anti-tumour activity. For a recent review of anti-cancer agents targeting signal transduction see de Bono and Rowinsky (2002) "Therapeutics targeting signal
20 transduction for patients with colorectal carcinoma", *Brit. Med. Bullet.* 64:227-254, which is incorporated herein by reference. This reference discusses IRESSA (formula V herein), TARCEVA (formula VI herein) and also PKI166 (4-(R)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrrolo[2.3-d]-pyrimidine) shown as formula IV herein,
25 GWS572016 (N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino)methyl}-2-furyl]-4-quinazolinamine) shown as formula III herein, Cl-1033 (4-(3-(chloro-4-fluoro-phenylamino)-7-(3-morpholin-4-yl-propoxy)-quinaxolin-6-yl)-acrylamide dihydrochloride])
30 shown as formula II herein, and EKB-569 (6-(4-dimethylamino)-but-2-enamido)-4-(3-chloro-4-fluoro-

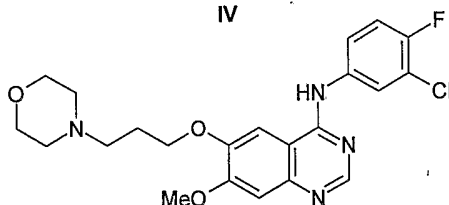
phenylamino)-7-ethoxy-quinolin-3-yl)-nitrile) shown as formula I herein as other EGFR tyrosine kinase inhibitors which are presently anti-cancer drug candidates undergoing development and trial.



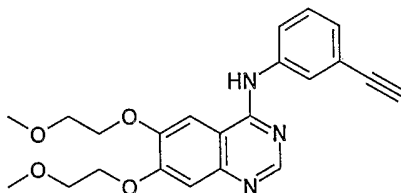
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IV



V



VI

Other known EGFR tyrosine kinase inhibitors include Tyrphostin AG-370 (Tyrphostin B7, 2-amino-4-(1H-indo-5'-yl)-1,1,3,3-tricyanobuta-1,3-diene, Sigma-Aldrich catalogue number T6443), and Tyrphostin AG1478 (N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinaxolinamine, Sigma-Aldrich catalogue number T4182) which are both specific for EGFR tyrosine kinase. Non-tyrphostin EGFR tyrosine kinase-specific inhibitors include: PD168393 (4-[(3-bromophenyl)amino]-6-acrylamidoquinazoline, Calbiochem catalogue number 513033); PD174265 (4-[(3-bromophenyl)amino]-6-propionylamidoquinazoline, Calbiochem catalogue number 513040); LFM-A12 (α -cyano- β -hydroxy- β -methyl-N-[4-(trifluoromethoxy)phenyl]propenamide, Calbiochem catalogue number 435302); PD153035 (4-[(3-bromophenyl)amino]-6,7-dimethoxyquinazoline, Calbiochem

catalogue number 234490); BPIQ-II (8-[3-bromophenyl)amino]-1H-imidazo[4,5-g]-quinazoline, Calbiochem catalogue number 203704); and BPIQ-1 (8-[(3-bromophenyl)amino]-3-methyl-3H-imidazo[4,5-g]-
5 quinazoline, Calbiochem catalogue number 203696).

The present invention includes the use of any EGFR tyrosine kinase receptor disclosed herein or in an incorporated reference.

In WO 96/33980, it is suggested that the disclosed
10 quinazolines may be effective in the treatment of vascular stenosis because of the origins of that disease in excessive cellular proliferation. In contrast, it has now been found that EGFR-specific kinase inhibitors are effective against a variety of cardiovascular
15 dysfunctions of non-proliferative origin, including hypertension, stroke and diabetes-induced cardiovascular dysfunction. Medicaments of the invention are believed to act by means of normalizing calcium homeostasis. The above-mentioned prior art does not disclose or
20 suggest any mechanism arising other than from excess proliferation or, in particular, arising from the mechanism herein proposed.

An in vitro assay for determining the ability of a given compound to inhibit the enzyme EGF receptor
25 tyrosine kinase is described in International Patent Specification No. WO 96/33980 at page 15, line 25 to page 17, line 3, the disclosure of which is incorporated herein by reference. The EGFR tyrosine kinase inhibitors used in accordance with the invention are preferably
30 EGFR-specific tyrosine kinase inhibitors. The term "EGFR-specific" in "EGFR-specific tyrosine kinase inhibitor" is used herein to refer to tyrosine kinase

inhibitors which have specificity for EGFR over other tyrosine kinase receptors. By way of example, "EGFR-specific" as used herein will normally refer to inhibitors for which the IC_{50} value for EGFR is less than 50%, and preferably less than 20% of that for at least one, preferably several, other receptor selected from HER2, HER3, HER4, PDGFR, TrK and lnsR. By way of example, some inhibitors for use in the invention may have an IC_{50} value for EGFR tyrosine kinase that is less than 50% of that for HER2 tyrosine kinase. However, an inhibitor which fails the above test will nonetheless be regarded as EGFR-specific if a person skilled in the art would normally regard it as such.

Assay conditions for determining specificity over HER2 may be found in Gazit et al., J.Med.Chem. (1991) 34, 1896 - 1907 under "Procedures for Receptor Autophosphorylation".

The tyrosine kinase inhibitor may be any substance that has inhibitory activity irrespective of its mechanism of activity and may for example be any tyrosine kinase inhibitor which acts to stop manufacture of the receptor or which otherwise interferes with the EGFR signalling pathway. As already described above, the tyrosine kinase inhibitor may be a discrete chemical which is capable of blocking the receptor kinase. The tyrosine kinase inhibitor may be a EGFR-specific antibody. Other examples of suitable classes of inhibitors are antisense oligonucleotides, ribozymes, DNA enzymes and siRNAs.

The following Example illustrates the invention. In the Example, reference is made to the accompanying drawings, in which:

- Fig. 1 is a graph of net weight loss in treated and untreated subjects;
- Fig 2. is a graph illustrating norepinephrine-induced vasoconstriction in subjects;
- 5 Fig. 3 is a graph illustrating endothelin-1-induced vasoconstriction in treated and untreated subjects;
- Fig. 4 is a graph illustrating angiotensin-II-induced vasoconstriction in treated and untreated subjects;
- 10 Fig. 5 is a graph illustrating carbachol-induced vasodilation in treated and untreated subjects; and
- Fig. 6 is a graph illustrating histamine-induced vasodilation in treated and untreated subjects.
- 15

Example

20 Step(a): Induction of diabetes

Female Wistar rats weighing 200-250g were used in this study. Diabetes was induced by a single intraperitoneal injection of 55mg/kg body weight streptozotocin (STZ) dissolved in citrate buffer (pH

25 4.5). Age-matched control rats were injected with the citrate buffer vehicle used to dissolve STZ. Body weight and basal glucose levels were determined prior to STZ injection, using an automated blood glucose analyzer (glucometer Elite XL). Blood glucose concentrations were

30 determined 48h after STZ injection. Rats with a blood glucose concentration above 300mg/dl were declared diabetic. The animals' body weights and the diabetic

state were re-assessed after 4 weeks just before sacrificing the animals.

Four groups of rats were used in this study. Group I was non-diabetic control rats (n=10). Group II was
5 STZ-diabetic rats without treatment (n=12). Group III was diabetic rats that received treatment with genistein (1.2 mg/kg/alt diem, n=8) and group IV was treated with AG1478 (1.2 mg/kg/alt diem, n=8). Treatment with the inhibitors of tyrosine kinases, genistein or AG1478, was
10 started on the same day as the induction of diabetes and continued every other day for four weeks.

Step (b): Isolation of the mesenteric vascular bed

The mesenteric beds were isolated carefully and
15 transferred into a Petri dish containing oxygenated Krebs' solution. The mesenteric artery was cannulated, using a polyethylene cannula and the mesenteric bed was placed in a warm water-jacketed chamber at 37°C. The preparation was perfused with Krebs' solution (at 37°C),
20 oxygenated with 95% oxygen and 5% carbon dioxide, delivered at a constant flow rate of 6 ml/min using a multichannel masterflex peristaltic pump. The composition of KH-solution was as follows (mM): NaCl (118.3), KCl (4.7), CaCl₂ (2.5), MgSO₄ (1.2), NaHCO₃ (25),
25 KH₂PO₄ (1.2) and glucose (11.2). Changes in perfusion pressure which reflect peripheral resistance were measured. Perfusion pressure was recorded via a pressure transducer connected to a Lectromed (Trade Mark of Lectromed UK Ltd) data logger. The preparation was
30 always allowed to equilibrate for at least 30 min. A bolus injection of norepinephrine (NE) (100 nmol) was

usually given at the beginning of the experiment as a test for tissue responsiveness.

Step (c) (i): Vasoconstriction studies

5 The vasoconstrictor responses of NE (10, 100 and 1000 nmol) and angiotensin II (0.1 and 1.0 nmol) were investigated in the perfused mesenteric vascular bed. Following the period of equilibration, successive doses of the agonists NE, endothelin-1 (ET-1) or angiotensin
10 II, were given at regular intervals to establish the vasoconstrictor responses (mmHg).

Step (c) (ii): Vasodilation Studies

15 The vasodilator responses of carbachol and histamine were investigated in the perfused mesenteric vascular bed. Following the period of equilibration, the perfused mesenteric bed was constricted by perfusion with Krebs' solution containing NE (10^{-5} M). After establishing a steady level of pre-contraction, successive doses of
20 carbachol (1 and 10 nmol) or histamine (10 or 100 nmol) were given at regular intervals. The vasodilator response is expressed as % of the pre-contraction induced by NE (10^{-5} M).

25 (±) Norepinephrine-bitartrate, streptozotocin, genistein, histamine, endothelin-1, angiotensin II, and carbachol for use in steps (a) to (c) above were obtained from Sigma Biochemicals. AG 1478 was purchased from Tocris Cookson Ltd., UK.

30 **Results**

Results were analyzed using Graph pad Prism software. Data are presented as Mean ± S.E. of 'n'

number of experiments. Mean values were compared using Student's t-test. Unless otherwise stated, the difference was considered to be significant when p value was less than 0.05. In Figs. 1 to 6, a single asterix (*) indicates values significantly different from non-diabetic controls and double asterix (**) indicates values significantly different from diabetic controls.

Hyperglycemia and Animals' body weights

10 Induction of diabetes by STZ resulted in a significant increase in blood glucose concentration. Hyperglycemia persisted in the diabetic animals and was 160 ± 24 mg/dl at four weeks as compared with 86 ± 6 mg/dl in the non-diabetic control animals. There was a
15 significant reduction of around 70g in the weights of STZ-diabetic rats (154 ± 6 g) compared to the non-diabetic control animals (224 ± 4 g) after 4 weeks diabetes whereas genistein or AG1478 treatment completely or significantly improved the weight of diabetic rats to 222 ± 6 g and
20 177 ± 8 g, respectively (see Fig. 1; Mean S.E., n=8-12.
* Significantly different from diabetic, # significantly different from diabetic-genistein-treated).

Vasoconstriction studies

25 The vasoconstrictor response to NE was significantly augmented in the perfused mesenteric vascular bed from STZ-diabetic rats to 85 ± 9 , 160 ± 7 mmHg and 177 ± 7 mmHg compared to 39 ± 7 , 106 ± 11 mmHg and 124 ± 6 mmHg in the non-diabetic control rats, at 10, 100 and 1000 nmol,
30 respectively (see Fig. 2; Mean $\pm$ S.E., n=8-12. * Significantly different from control, ** significantly different from diabetic).

The potentiated vasopressor activity of NE was significantly ($p < 0.05$) attenuated by 4-week treatment of diabetic rats with the tyrosine kinase inhibitors (genistein and AG1478) (see Fig. 2). NE-induced vasoconstriction in the perfused mesenteric bed of genistein-treated animals was 97 ± 18 mmHg and 119 ± 23 mmHg to NE at 100 and 1000 nmol, respectively. Similarly, the vasoconstrictor response to NE in AG1478-treated diabetic perfused mesenteric bed was 91 ± 23 mmHg and 114 ± 11 mmHg at 100 and 1000 nmol, respectively (see Fig. 2).

ET-1 induced vasoconstriction was also significantly ($p < 0.05$) augmented in the diabetic rat perfused mesenteric bed as shown by Fig. 3. ET-1 induced vasoconstriction was 138 ± 14 and 198 ± 7 mmHg in the diabetic mesenteric bed compared to 75 ± 14 and 99 ± 9 mmHg at 0.1 and 1.0 nmol, respectively, in group I non-diabetic controls (see Fig. 3; Mean $\pm$ S.E., $n = 8-12$. *Significantly different from control, ** significantly different from diabetic). Treatment of the diabetic rats with genistein normalized the vasoconstrictor response to ET-1 (0.1 and 1.0 nmol) to 66 ± 18 and 157 ± 26 mmHg. Similarly, AG1478-treatment also produced a significant attenuation of ET-1 induced vasoconstriction (0.1 nmol) to 80 ± 25 mmHg (see Fig. 3).

A relatively modest vasoconstrictor response to angiotensin II (0.1 and 1.0 nmol) was observed in the perfused mesenteric bed yet it was significantly increased in the diabetic state compared to the non-diabetic control rats (see Fig. 4; Mean $\pm$ S.E., $n = 8-12$. *Significantly different from control, ** significantly different from diabetic). Treatment of diabetic rats with the tyrosine kinase inhibitors (genistein or AG1478)

significantly reduced angiotensin II-induced vasoconstriction, to levels similar to those observed in non-diabetic controls (Fig. 4).

Vasodilation Studies

5 The vasodilator response to carbachol was significantly reduced in the perfused mesenteric vascular bed from STZ-diabetic rats to $44 \pm 7\%$ and $59 \pm 6\%$ compared to $78 \pm 5\%$ and $100 \pm 6\%$ in the non-diabetic control rats, at 1 and 10 nmol, respectively, as illustrated by Fig. 5.

10 Treatment of the diabetic rats with genistein or AG1478 prevented the diabetes-induced attenuation of carbachol-mediated vasodilation (see Fig. 5; Mean $\pm$ S.E., $n=8-12$. *Significantly different from control, ** significantly different from diabetic.) The vasodilator response to

15 histamine (100nmol) was significantly reduced in the perfused mesenteric vascular bed from STZ-diabetic rats to $56 \pm 12\%$ compared to $97 \pm 12\%$ in the non-diabetic control rats (see Fig. 6; Mean $\pm$ S.E., $n=8-12$. *Significantly different from control, ** significantly different from

20 diabetes). Treatment of the diabetic rat with genistein or AG1478 prevented the diabetes-induced reduction in histamine-induced vasodilation (see Fig. 6) The vasodilator responses to histamine (10 nmol) was $59 \pm 14\%$ in genistein-treated diabetic rats compared to $17 \pm 4\%$ in

25 diabetes. Histamine-induced vasodilation (100 nmol) was normalized to a value of $94 \pm 8\%$ in AG1478-treated diabetic rats (see Fig. 6).

Discussion

30 Diabetes mellitus affects the structural and functional integrity of many organ systems including the cardio-vasculature. Diabetes-induced abnormalities in

vascular function are now well established despite conflicting reports on whether the vascular responsiveness is enhanced or attenuated for a given vasoactive agonist. In the perfused mesenteric vascular bed from STZ-induced diabetic rats used in the present study, there is observed an exaggerated response to vasoconstrictors, Ang II, NE and ET-1, and an attenuated response to the vasodilators, carbachol and histamine. Further, it is demonstrated for the first time that EGFR TK is an essential mediator in the development of vascular dysfunction during diabetes as its chronic inhibition as demonstrated herein normalized both the altered vasoconstrictor (Figs. 2 - 4) and vasodilator responses (Figs. 5 - 6) in the perfused mesenteric vascular bed of STZ-diabetic rats. In addition, a significant prevention of weight loss was observed following treatment of animals with inhibitors of tyrosine kinases, with complete abrogation of weight loss upon genistein treatment (see Fig. 1), implying that tyrosine kinases, including EGFR, are also either directly involved in the pathways that lead to weight loss during diabetes or alternatively their inhibition may influence weight gain subsequent to correction of vascular dysfunction. As such it provides valuable insight into the signalling pathways involved in altered vascular response and weight loss.

Here we show that EGFR signalling is also important in mediating diabetic vascular dysfunction. However, the precise mechanisms responsible for EGFR pathway-induced vascular damage in diabetes are not known. It is possible that EGFR-led changes in calcium homeostasis may be important. It is known that Ca^{2+} is a ubiquitous

intracellular messenger. It is also known that abnormalities in calcium homeostasis are responsible for exaggerated vascular reactivity in cardiovascular diseases such as hypertension and diabetes. For example, it has been shown that enhanced contractile responses of the diabetic aortae and mesenteric arteries to α_1 -agonists are due to altered levels of intra- and extra-cellular calcium. The hypothesis that tyrosine kinases, including EGFR tyrosine kinase, are involved in mediating vascular dysfunction via modulation of calcium homeostasis that leads to altered responsiveness to vasoconstrictors/ vasodilators is consistent with several recent reports. It has been shown in aortic strips that tyrosine kinases contribute to agonist-induced vascular smooth muscle contraction mediated through increased calcium sensitivity and increased calcium entry (Carter and Kanagy, (2001), Hypertension 38, 521 (abstr.)). In addition, it has been shown that 20-HETE, a potent vasoconstrictor produced by metabolism of arachidonic acid (AA) via CYP-450, activates the MAPK-pathway in renal arterioles and that activation of a TK contributes to the inhibitory effects of 20-HETE on K^+ -channel activity and its vasoconstrictor effects (Sun et al., (1999) Hypertension, 33, 414-418). Our data are also consistent with the recent hypothesis of Roman (2002), Physiol. Rev. 82, 131-185, that 20-HETE may lead to enhanced calcium sensitivity via activation of the EGFR/shc/Grb2/sos/Ras pathway as inhibition of EGFR tyrosine kinase activity normalized vascular function. Furthermore, it has been suggested that inhibition of CYP-450, the enzyme responsible for producing 20-HETE, also normalizes the altered vascular reactivity in

diabetes, further implicating this metabolite of AA as an important mediator of diabetic vascular dysfunction. The data now obtained by the inventor, taken together those earlier findings, apparently supports the role of

5 tyrosine kinases including EGFR in altering calcium homeostasis, possibly via signalling pathways involving 20-HETE, that subsequently results in abnormal vascular functions in diabetes.

The above results also indicate significance of EGFR

10 inhibition in mediating weight gain in diabetic animals. It has previously been reported that sustained administration of EGF, a ligand for EGFR, leads to reduced fat mass in rats despite an unaltered food intake (Kristensen et al., (1998), Horm. Res. 50, 292-296;

15 Pedersen et al., (2000), Biochem. Biophys. Res. Commun. 279, 914-919). Those authors proposed that EGF administration reduced body mass by enhancing activation of mitochondrial uncoupling proteins (UCP2 and UCP3) involved in increased energy expenditure (Pedersen et

20 al., 2000) that would ultimately result in induction of lipolysis. It is believed that the attenuation of weight loss demonstrated herein due to treatment with AG1478 or genistein during development of diabetes may be occurring via inhibition of EGF/EGFR/UCP2,3-pathway and through

25 inhibition of lipolysis. Furthermore, lipolysis, or more specifically, non-esterified fatty acid (NEFA) products of lipolysis, have an adverse effect on vascular reactivity, (Egan and Greene, (1999), Prostaglandin Lenkot. Essent. Fatty Acids, 60, 411-420) possibly via

30 modulation of protein kinase C (PKC) activity and calcium homeostasis. NEFAs can lead to activation of PKC and have also been reported to directly activate EGFR

(Vacaresse et al., (1999), Circ. Res. 85, 892-299). The inventors' findings now suggest that EGFR-mediated signalling may be a key link between signal transduction pathways leading to weight loss and vascular dysfunction.

5 The role of RTKs, as exemplified by EGFR phosphorylation by its ligand EGF, may lead to vascular damage via altered calcium homeostasis and increased calcium sensitivity via a shc/Grb2/sos/CaMKII-mediated pathway. Also EGF-mediated phosphorylation may be a key
10 signalling pathway leading to weight loss in diabetes. This may be mediated via an EGF/EGFR induction of the mitochondrial coupling proteins UCP2,3 leading to increased energy expenditure and increased lipolysis. The latter may also contribute to vascular damage as it
15 leads to production of NEGAs that can directly activate EGFR. Alternatively, NEFAs may induce vascular dysfunction via activation of PKC.

 In summary, it is shown herein that treatment with genistein or AG1478 produced a significant normalization
20 of the altered agonist-induced vasoconstrictor and vasodilator responses as well as preventing marked weight loss that normally occurs during diabetes. Thus, the data herein suggest that signalling via TKs, including EGFR, is an essential component in the development of
25 diabetic vascular dysfunction and may also play an important role in signalling pathways leading to weight loss during diabetes. In accordance with the invention, therefore, the use of EGFR tyrosine kinase inhibitors offers a novel and promising treatment for vascular
30 complications and weight loss in diabetic patients, and possibly also in non-diabetic patients.

Claims

1. Use of an EGFR tyrosine kinase inhibitor in the manufacture of a medicament for use in the therapeutic or
5 prophylactic treatment of cardiovascular dysfunction.

2. Use according to claim 1, in which the cardiovascular dysfunction is non-proliferative in origin.

3. Use according to claim 1 or claim 2 in which
10 the cardiovascular dysfunction is characterised by abnormal vascular reactivity.

4. Use according to any one of claims 1 to 3, wherein the cardiovascular dysfunction is a diabetes-induced cardiovascular dysfunction.

15 5. Use according to any one of the preceding claims in which the cardiovascular dysfunction is hypertension.

6. Use according to any one of claims 1 to 4, wherein the cardiovascular dysfunction is stroke.

20 7. Use according to any one of claims 1 to 4, wherein the cardiovascular dysfunction is retinopathy.

8. Use according to any one of claims 1 to 4, wherein the cardiovascular dysfunction is nephropathy.

9. Use according to any one of claims 1 to 4,
25 wherein the cardiovascular dysfunction is male erectile dysfunction.

10. Use according to any one of the preceding claims, in which the inhibitor is a quinazoline derivative.

30 11. Use according to any one of the preceding claims, in which the inhibitor is an anilino quinazoline derivative.

12. Use according to claim 11 in which the inhibitor is a 4-(3',4' dihaloanilino)-7-alkoxy-6-(morpholinopropoxy) quinazoline or a physiologically acceptable salt thereof.

5 13. Use according to claim 11, in which the inhibitor is 4-(3'-chloro-4'-fluoro anilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline ("IRESSA") or a physiologically acceptable salt thereof.

10 14. Use according to claim 11, in which the inhibitor is [6,7-bis(2-methoxyethoxy)quinazolin-4-yl]-(3-ethynylphenyl)amine ("TARCEVA") or a physiologically acceptable derivative thereof.

15 15. Use according to any one of claims 1 to 9, in which the inhibitor is an antibody having EGFR specificity.

16. Use according to any of the one of the proceeding claims, in which the inhibitor is an EGFR-specific tyrosine kinase inhibitor.

20 17. Use of a tyrosine kinase inhibitor in the manufacture of a medicament for use in the therapeutic or prophylactic treatment of diabetes-induced cardiovascular disease.

25 18. An EGFR tyrosine kinase inhibitor for use in the therapeutic or prophylactic treatment of cardiovascular dysfunction.

30 19. A EGFR tyrosine kinase inhibitor according to claim 17, wherein the EGFR tyrosine kinase inhibitor is as defined in accordance with any one of claims 10 to 16 and/or the cardiovascular dysfunction is defined in accordance with any one of claims 2 to 9.

20. A method for alleviating cardiovascular dysfunction of a subject, comprising administering to

said subject a therapeutically or prophylactically effective amount of an EGFR tyrosine kinase inhibitor.

21. A method according to claim 20, wherein the EGFR tyrosine kinase inhibitor is as defined in
- 5 accordance with any one of claims 10 to 16 and/or the cardiovascular dysfunction is defined in accordance with any one of claims 2 to 9.

Figure 1

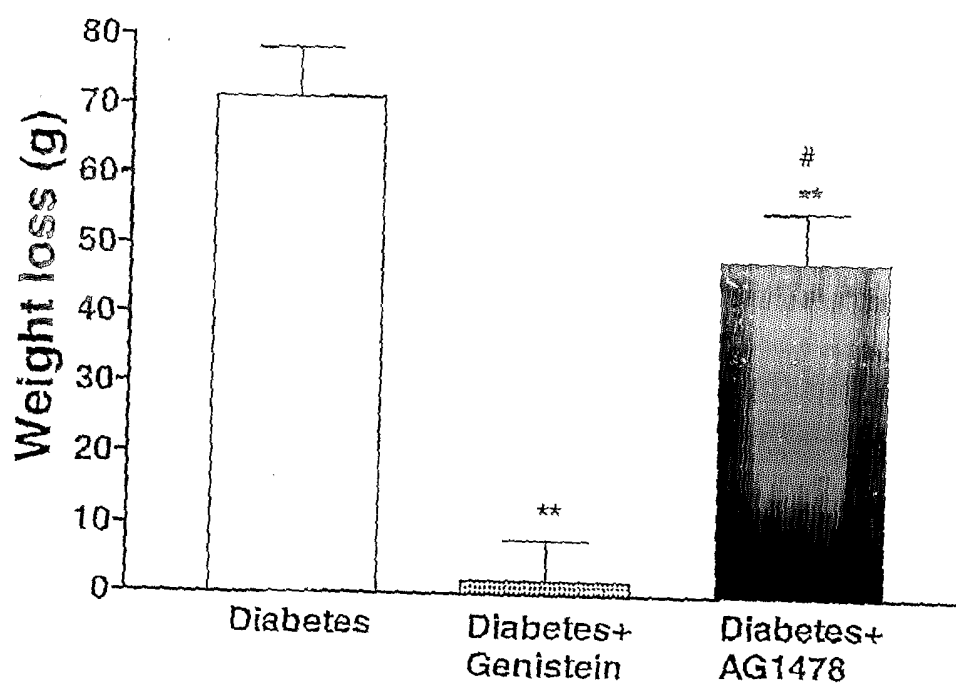


Figure 2

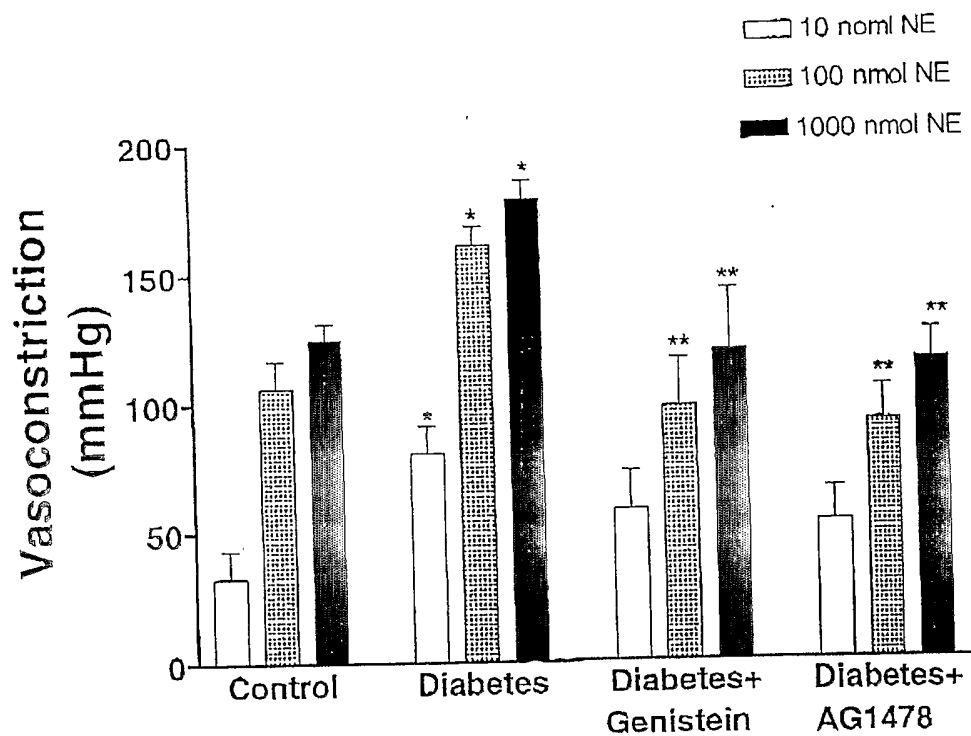


Figure 3

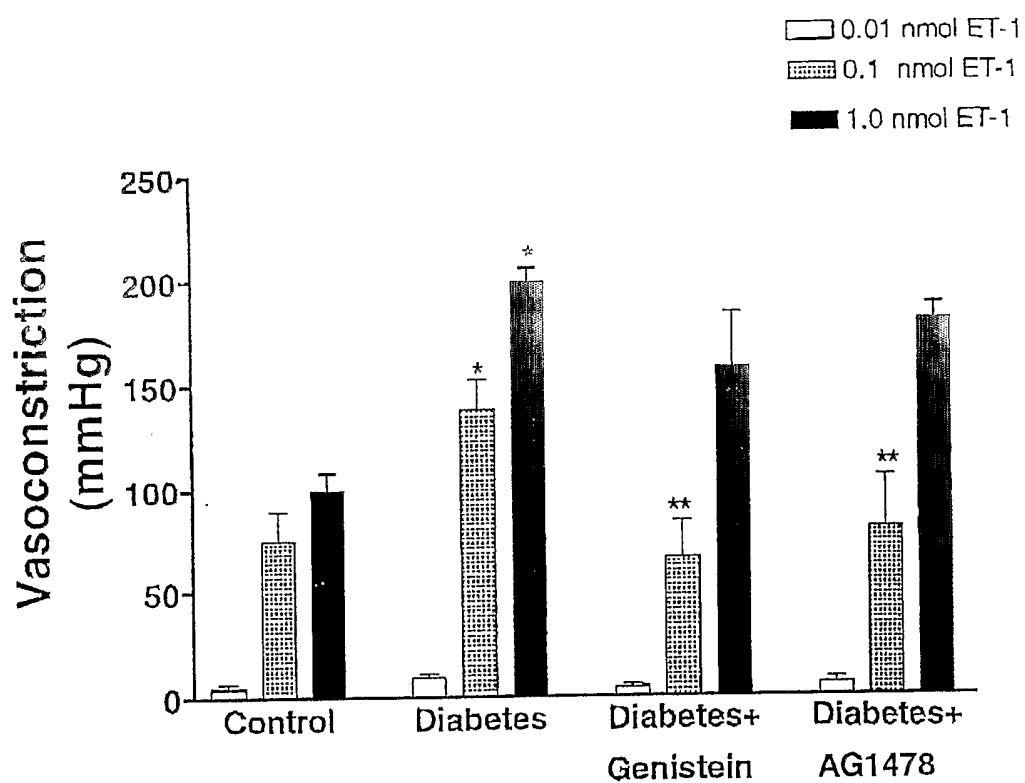


Figure 4

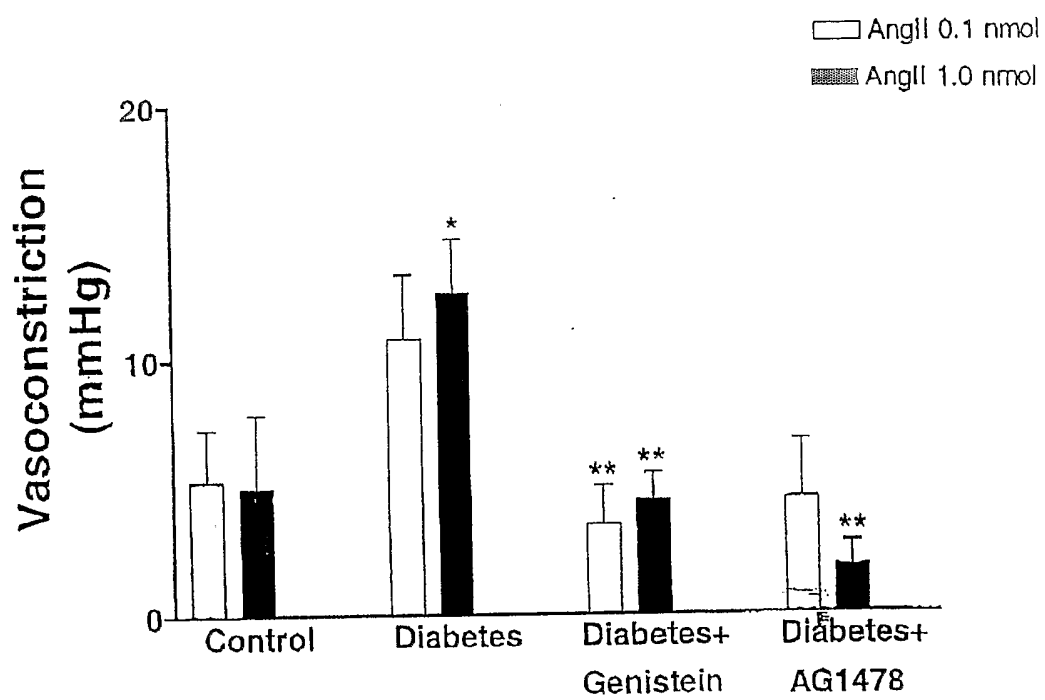


Figure 5

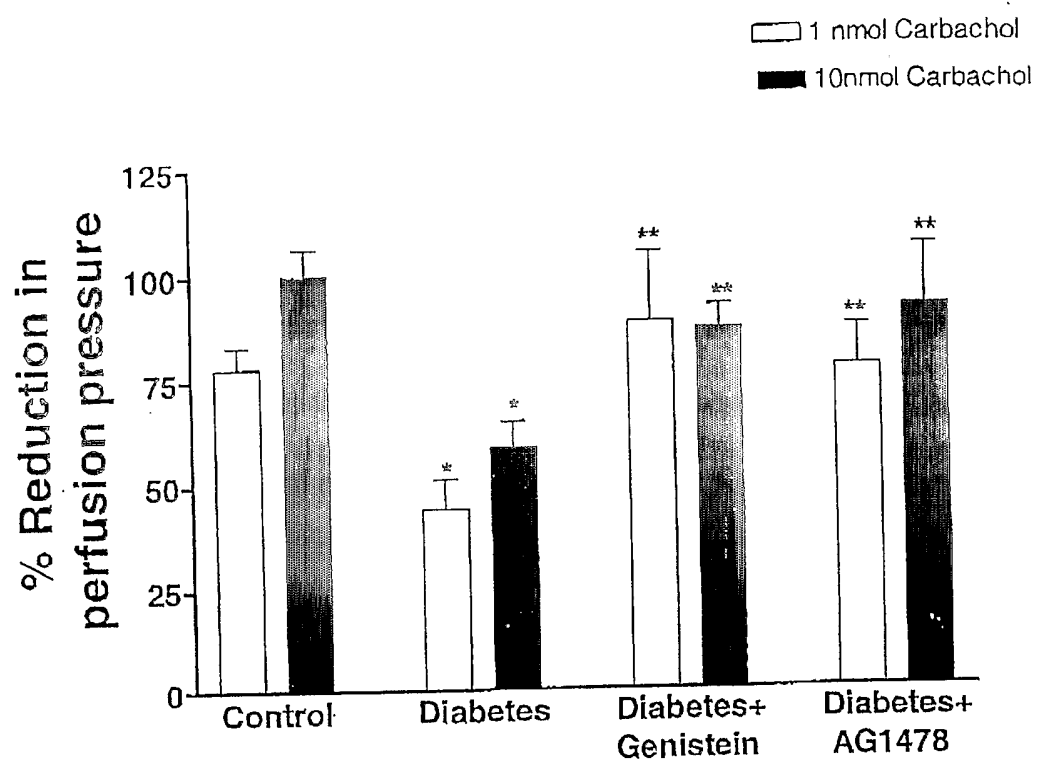


Figure 6

