R59949 has a slightly greater potency than R59022, it produces a similar, *incomplete* inhibition of PA formation following challenge with thrombin or membrane-permeable diacylglycerols and this is not associated with increased phosphorylation of a substrate for PKC.

The study therefore offers no explanation for the potentiation of thrombin-induced secretion by R59949. However, there are a number of substrates for PKC in platelets, the phosphorylation of which cannot be readily quantified on a one-dimensional gel e.g. [12]. It is possible that phosphorylation of one of these is potentiated by R59949 and that this protein is involved in secretion. (The role of the 47 kDa protein in platelets is unclear and no direct evidence is available to suggest that it plays a role in secretion.)

In summary, we have investigated the action of a novel inhibitor of DG-kinase, R59949. This agent was found to produce partial inhibition of formation of phosphatidic acid in human platelets challenged with thrombin, DC8 or OAG. However, this effect was not associated with enhanced phosphorylation of a 47 kDa protein, a known substrate for protein kinase C. We therefore believe that this compound does not represent a major advance on its earlier prototype, R59022.

Acknowledgements—B.R.L. was supported by an F.P.I. grant from the Ministerio de Educacion y Ciencia (Spain), T.W. is in receipt of a B.H.F. studentship and S.P.W. is a Royal Society Universal Research Fellow.

Department of Pharmacology
South Parks Road
Oxford OX1 3QT
U.K.

BELÉN RODRÍGUEZ-LIÑARES*
TREVOR WALKER†
STEVE WATSON†

REFERENCES

1. Berridge MJ and Irvine RF, Inositol phosphates and cell signalling. *Nature* **341**: 197–205, 1989.
2. Rink TJ and Hallam TJ, What turns platelets on. *Trends Biochem Sci* **8**: 215–219, 1984.
3. Lapetina EG and Watson SP, The relative importance of inositol 1,4,5-trisphosphate, 1,2-diacylglycerol and phosphatidic acid in mediating human platelet activation by thrombin. *Proc IUPHAR (London, 1985)* **1**: 127–131, 1984.
4. Nishizuka Y. Calcium phospholipid turnover and transmembrane signalling. *Phil Trans R Soc Lond B* **302**: 101–112, 1983.
5. Rink TJ, Sanchez A and Hallam TJ, Agonist selectivity and second-messenger concentration in Ca^{++}-mediated secretion. *Nature* **305**: 317–319, 1983.
6. Watson SP and Lapetina EG, 1,2-Diacylglycerol and phorbol ester inhibit agonist-induced production of inositol phosphates in human platelets; possible implications for negative feedback regulation of inositol phospholipid hydrolysis. *Proc Natl Acad Sci USA* **82**: 2623–2626, 1985.
7. Watson SP and Hambleton S, Phosphorylation-dependent and -independent pathways of platelets aggregation. *Biochem J* **258**: 479–485, 1989.
8. de Chaffoy de Courcelles D, Roevens P, Belle HV, Kennis L, Somers Y and de Clerck F, The role of endogenously formed diacylglycerol in the propagation of termination of platelet activation. *J Biol Chem* **264**: 3274–3285, 1989.
9. Watson SP, Ganong BR, Bell RM and Lapetina EG, 1,2-Diacylglycerols do not potentiate the action of phospholipases A_2 and C in human platelets. *Biochem Biophys Res Commun* **121**: 386–391, 1984.
10. Nunn DL and Watson SP, A diacylglycerol kinase inhibitor, R59022, potentiates thrombin-induced platelet aggregation and secretion. *Biochem J* **243**: 809–813, 1987.
11. de Chaffoy de Courcelles D, Roevens P and Van Belle H, R59022, a diacylglycerol kinase inhibitor. *J Biol Chem* **260**: 15762–15770, 1985.
12. Bourguignon LYW, Walker G and Bourguignon GJ, Phorbol ester-induced phosphorylation of a transmembrane glycoprotein (GP 180) in human blood platelets. *J Biol Chem* **260**: 11775–11780, 1985.
13. Tyers M, Rachubinski RA, Stewart MI, Varrichio A M, Shorr RGL, Haslam RJ, and Harley CB, Molecular cloning and expression of the major protein kinase C substrate of platelets. *Nature* **333**: 470–473, 1988.

* On leave from Departamento de Farmacologia, Universidad de Santiago de Compostela, Avda. de las Ciencias s/n, Santiago de Compostela, Spain.
† Authors for correspondence.

Biochemical Pharmacology, Vol. 41, No. 5, pp. 838–841, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Assessment of the drug inhibitor specificity of the human liver 4-methylumbelliferone UDP-glucuronosyltransferase activity

(*Received* 10 *August* 1990; *accepted* 6 *November* 1990)

Conjugation with glucuronic acid, a process catalysed by the enzyme UDP-glucuronosyltransferase (UDPGT), is responsible for the metabolism of many clinically used drugs. It is now well established that UDPGT exists as a multi-gene family in the rat and that individual UDPGT rat liver isozymes tend to differ in terms of regulation and xenobiotic substrate specificity [1]. The multiplicity of human liver UDPGT is also now accepted on the basis of results from microsomal kinetic and inhibitor studies [2–6], the cloning of UDPGT cDNAs [7–9] and the isolation of purified enzymes [10]. However, despite the recognition of the heterogeneity of human liver UDPGT, little is known about isozyme substrate specificity, particularly with respect to therapeutic drugs.

One of the human liver microsomal UDPGT activities characterized to date [4] is that associated with the glucuronidation of the xenobiotic substrate 4-methylumbelliferone (4MU). Available evidence suggests the 4MU-UDPGT activity comprises at least two closely related isozymes with similar kinetic properties and broadly comparable chemical substrate specificities [4, 8, 10]. To determine the possible importance of the isozymes comprising

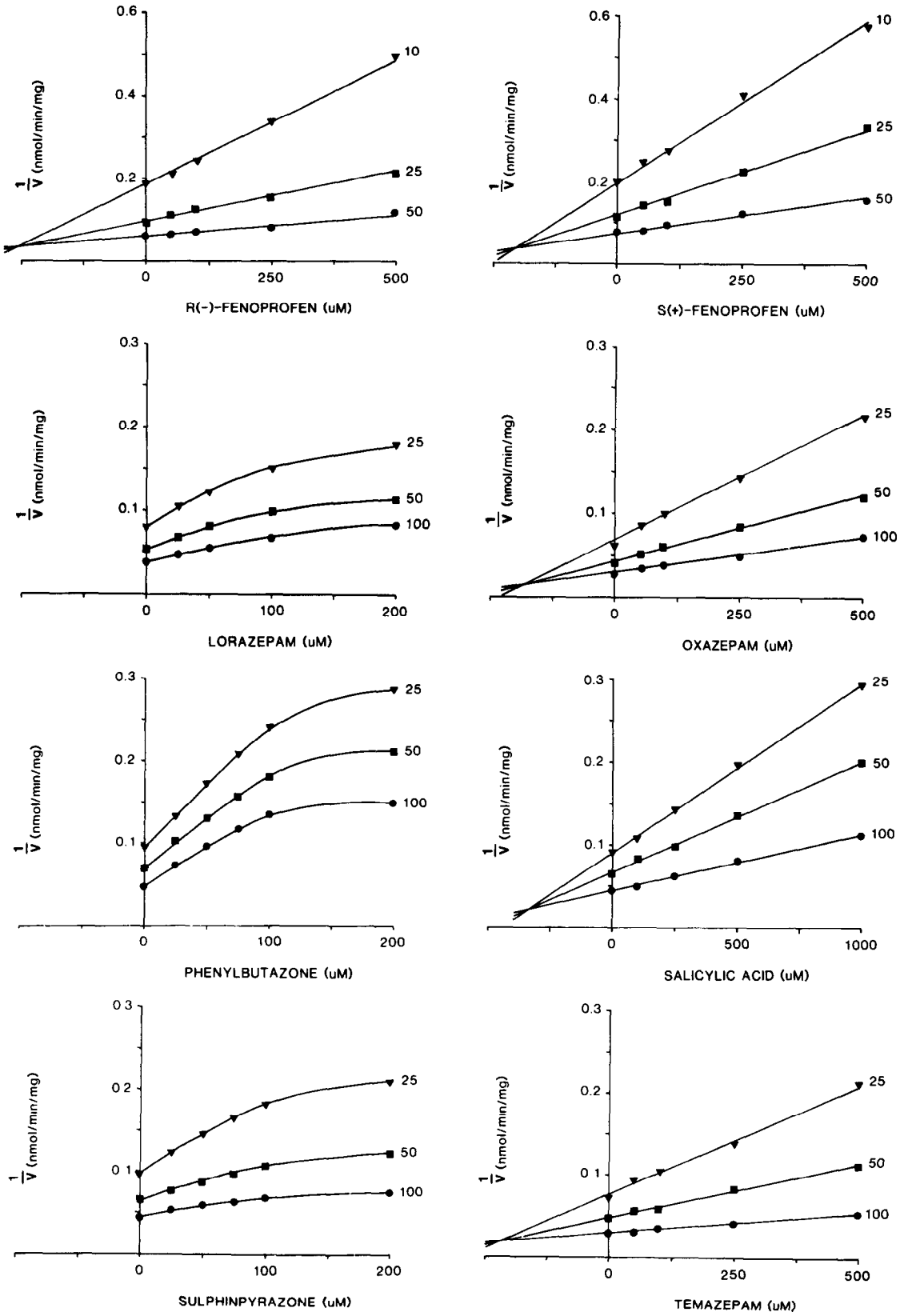

Fig. 1. Dixon plots for the inhibition of 4-methylumbelliferone glucuronidation in human liver microsomes by *R*- and *S*-fenoprofen, lorazepam, oxazepam, phenylbutazone, salicylic acid, sulphinpyrazone and temazepam. Concentrations of 4-methylumbelliferone are indicated on the right hand side of individual plots.

the 4MU-UDPGT activity in drug metabolism, a number of clinically used drugs which are known to be glucuronidated *in vivo* were screened for their ability to inhibit 4MU glucuronidation in human liver microsomes.

Materials and Methods

Chloramphenicol, clofibric acid, 4MU, paracetamol, probenecid, salicylamide and salicylic acid were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Lorazepam, oxazepam and temazepam were obtained from Wyeth Pharmaceuticals (Sydney, Australia), morphine from Glaxo Australia (Melbourne, Australia), phenylbutazone and sulphinpyrazone from Ciba-Geigy (Sydney, Australia), and valproic acid from ICN Pharmaceuticals (Plainview, NY, U.S.A.). Racemic ibuprofen and ketoprofen were supplied by Boots Australia (Sydney, Australia) and May and Baker (Melbourne, Australia), respectively. The individual enantiomers (*R* and *S*) of fenoprofen and naproxen were obtained from the late Dr P. J. Meffin (Flinders Medical Centre, Adelaide). Incubations were performed using detergent activated human liver microsomes as previously described [4] and contained 4MU (50 μM), UDP–glucuronic acid (5 mM), microsomal protein (10 μg), Brij 58 (0.0003%, w/v), $MgCl_2$ (5 mM) and Tris–HCl (0.1 M, pH 7.4). Formation of product (4MU-β-D-glucuronide) was determined fluorometrically [4]. In the screening studies, incubations were performed using microsomes from three livers (F5–F7; see Ref. 11 for donor details) in the presence and absence of each of the drugs listed above; the final concentration of added drug in the incubations was 250 μM. Each drug screened was tested for interference with 4MU-glucuronide fluorescence. Where inhibition was observed, full kinetic studies were performed with a range of 4MU and drug concentrations (see Fig. 1) using pooled microsomes from livers F5–F7 to determine the type of inhibition. In those cases where inhibition was shown to be competitive in nature, apparent K_i values were determined according to the procedure of Dixon [4, 12].

Results and Discussion

The results are summarized in Table 1. Of the drugs screened, paracetamol, chloramphenicol, clofibric acid, *R*,*S*-ibuprofen, *R*,*S*-ketoprofen, *R*- and *S*-naproxen, morphine, probenecid, salicylamide and valproic acid all caused <10% inhibition of 4MU glucuronidation. At the relative 4MU (50 μM) and drug (250 μM) concentrations used, calculations [13] based on the known apparent K_m for 4MU [4] indicate that any potential inhibitor with an apparent K_i <2 mM should cause >10% inhibition of microsomal 4MU metabolism. Most glucuronidated xenobiotics investigated to date [4, 5] appear to have K_m values <2 mM, although a recent study [6] has shown the apparent K_m for paracetamol glucuronidation in human liver microsomes is approximately 7 mM. However, even when the concentration of added paracetamol in microsomal incubations was raised as high as 5 mM, significant inhibition did not occur (data not shown). Thus, it would appear that paracetamol, chloramphenicol, clofibric acid, ibuprofen, ketoprofen, morphine, probenecid, salicylamide and valproic are not substrates for the isozymes comprising in the 4MU-UDPGT activity.

In contrast, *R*- and *S*-fenoprofen, oxazepam, salicylic acid and temazepam were all competitive inhibitors of the 4MU-UDPGT activity (Table 1 and Fig. 1) and may therefore be alternative substrates for these transferases. Interestingly, the apparent K_i values calculated for *R*- and *S*-fenoprofen were similar suggesting that the interaction of the fenoprofen enantiomers with the 4MU-UDPGT activity is non-stereoselective. Lorazepam, phenylbutazone and sulphinpyrazone exhibited atypical inhibition kinetics.

Table 1. Inhibitory effects of various drugs on 4-methylumbelliferone glucuronidation by human liver microsomes

Inhibitor*	% Remaining activity†	Apparent K_i (μM)‡
Chloramphenicol	101 ± 4	—
Clofibric acid	103 ± 5	—
R-Fenoprofen	57 ± 6	253
S-Fenoprofen	55 ± 4	220
R,*S*-Ibuprofen	92 ± 4	—
R,*S*-Ketoprofen	92 ± 5	—
Lorazepam	28 ± 6	Atypical
Morphine	98 ± 5	—
R-Naproxen	92 ± 6	—
S-Naproxen	95 ± 3	—
Oxazepam	43 ± 5	188
Paracetamol	96 ± 4	—
Phenylbutazone	12 ± 5	Atypical
Probenecid	103 ± 4	—
Salicylamide	90 ± 2	—
Salicylic acid	72 ± 5	367
Sulphinpyrazone	44 ± 2	Atypical
Temazepam	52 ± 6	217
Valproic acid	98 ± 3	—

* 4-Methylumbelliferone concentration, 50 μM; inhibitor concentration, 250 μM.

† Mean ± SD from three livers (F5–F7).

‡ Single result using pooled microsomes from three liver (F5–F7). Atypical signifies atypical inhibition.

This may indicate that these drugs differentially inhibit the isozymes comprising the 4MU-UDPGT activity.

In summary, the data suggest that the 4MU-UDPGT activity of human liver microsomes probably contributes to the glucuronidation of a limited number of clinically used drugs. However, confirmation of this ultimately requires studies to be performed with purified isozymes, cDNAs expressed in cell culture, or specific inhibitory antibodies.

Acknowledgement—This work was supported by a grant from the National Health and Medical Research Council of Australia.

Department of Clinical Pharmacology
Flinders Medical Centre
Bedford Park
Adelaide
SA 5042, Australia

JOHN O. MINERS*
KAREN J. LILLYWHITE

REFERENCES

1. Burchell B and Coughtrie MWH, UDP-Glucuronosyltransferases. *Pharmacol Ther* **43**: 261–289, 1989.
2. Bock KW, Brunner G, Hoensch H, Huber E and Josting D, Determination of UDP-glucuronyltransferase in needle biopsy specimens of human liver. *Eur J Clin Pharmacol* **14**: 367–373, 1978.
3. Bock KW, Lilienblum W and von Bahr C, Studies of UDP-glucuronyltransferase activities in human liver microsomes. *Drug Metab Dispos* **12**: 93–97, 1984.
4. Miners JO, Lillywhite KJ, Matthews AP, Jones ME and Birkett DJ, Kinetic and inhibitor studies of 4-methylumbelliferone and 1-naphthol glucuronidation in human liver microsomes. *Biochem Pharmacol* **37**: 665–671, 1988.
5. Miners JO, Lillywhite KJ and Birkett DJ, *In vitro* evidence for the involvement of at least two forms of human liver UDP-glucuronosyltransferase in morphine

* To whom correspondence should be addressed.

3-glucuronidation. *Biochem Pharmacol* **37**: 2839–2845, 1988.
6. Miners JO, Lillywhite KJ, Yoovathaworn K, Pongmarutai M and Birkett DJ, Characterisation of paracetamol UDP-glucuronosyltransferase activity in human liver microsomes. *Biochem Pharmacol* **40**: 595–600, 1990.
7. Fournel-Gigleux S, Jackson MR, Wooster R and Burchell B, Expression of a human liver cDNA encoding a UDP-glucuronosyltransferase catalysing the glucuronidation of hyodeoxycholic acid in cell culture. *FEBS Lett* **243**: 119–122, 1989.
8. Harding D, Fournel-Gigleux S, Jackson MR and Burchell B, Cloning and substrate specificity of a human liver UDP-glucuronosyltransferase expressed COS-7 cells. *Proc Natl Acad Sci USA* **85**: 8381–8385, 1988.
9. Jackson MR, McCarthy LR, Harding D, Wilson S, Coughtrie MWH and Burchell B, Cloning of a human liver microsomal UDP-glucuronosyltransferase cDNA. *Biochem J* **242**: 581–588, 1987.
10. Irshaid YM and Tephly TR, Isolation and purification of two human liver UDP-glucuronosyltransferases. *Mol Pharmacol* **31**: 27–34, 1987.
11. Robson RA, Matthews AP, Miners JO, McManus ME, Meyer UA, Hall P and Birkett DJ, Characterisation of theophylline metabolism in human liver microsomes. *Br J Clin Pharmacol* **24**: 293–300, 1987.
12. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55**: 170–171, 1953.
13. Segel I, *Enzyme Kinetics*, p. 106. J. Wiley & Sons, New York, 1975.

Biochemical Pharmacology. Vol. 41, No. 5, pp. 841–844, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

The atriopeptidase inhibitor (±)candoxatrilat reduces the clearance of atrial natriuretic factor in both intact and nephrectomized rats: evidence for an extra-renal site of action

(*Received* 28 *August* 1990; *accepted* 13 *November* 1990)

Atrial natriuretic factor (ANF*) is a 28 amino acid peptide with natriuretic, diuretic and vasodilator properties [1]. On exogenous administration it is rapidly cleared from the circulation resulting in a short *in vivo* half-life [2]. The peptide is degraded by the zinc-dependent neutral endopeptidase (EC 3.4.24.11, atriopeptidase) *in vitro* [3, 4], and several groups have recently reported that inhibitors of this enzyme (atriopeptidase inhibitors, API) potentiate the renal and vasodilator effects of ANF in animal models [5, 7].

(±)Candoxatrilat (*cis* - 4 - {[2 - carboxy - 3 - (2 - methoxyethoxy)propyl]- 1- cyclopentanecarbonylamine}- 1- cyclohexane carboxylic acid, UK-69,578) is a potent, selective inhibitor of atriopeptidase (K_i 36 nM) which evokes a doubling of plasma ANF levels, and increases sodium excretion and urine flow following acute administration to rodents [8]. A similar maximal increase in plasma ANF levels associated with a rise in sodium excretion (with no effect on potassium excretion) and fall in plasma renin activity is observed following acute administration of the compound to man [9]. This profile resembles that elicited by low-level ANF infusions when plasma ANF levels are increased by a similar degree [10].

The precise mechanism of action of (±)candoxatrilat *in vivo* is currently the subject of further investigation and preliminary studies have demonstrated that the natriuretic and diuretic responses to (±)candoxatrilat are blocked by ANF antiserum [11]. In the present study we sought to determine the effects of (±)candoxatrilat on the clearance of ANF in anaesthetized rats, and to define the kinetics of the clearance processes. Furthermore, since the kidney is a major site of ANF clearance [12] and atriopeptidase is found in abundance in this tissue [13], we examined whether nephrectomy altered the profile of activity of (±)candoxatrilat.

* Abbreviations: ANF, atrial natriuretic factor; API, atriopeptidase inhibitor; *AUC*, area under the plasma concentration curve; CL_p, plasma clearance; ^{125}I-ANF, (3-[^{125}I]iodotyrosyl^{28}) rat ANF; V_d, volume of distribution.

Materials and Methods

Disappearance of ^{125}I-ANF in anaesthetized rats. Male Sprague–Dawley rats (375 g) were anaesthetized with Inactin (120 mg/kg i.p.) and catheters placed in the trachea, carotid artery and femoral vein. After a 60 min stabilization period 2.5 μCi (12 ng/kg) of ^{125}I-ANF (Amersham International, U.K.) was injected i.v. in 50 μL saline, and arterial blood samples (0.3 mL) taken at 15, 30, 60, 90, 120, 180, 240 and 300 sec into syringes containing EDTA (1 mg/mL) and aprotinin (1000 kI.U./mL). Samples were centrifuged immediately to obtain plasma, which was mixed with 3 volumes of 4% (v/v) acetic acid and stored on ice prior to extraction. One hour after the initial injection of ^{125}I-ANF, animals were dosed intravenously with saline vehicle or (±)candoxatrilat (3 mg/kg) followed 10 min later by a second injection of 2.5 μCi ^{125}I-ANF, and a further eight blood samples obtained and processed as described above. Aliquots (300 μL) of plasma in acetic acid were loaded onto Sep-Pak C18 columns (Waters, U.K.), and the columns were washed with 10 mL distilled water followed by 10 mL 4% (v/v) acetic acid. Columns were eluted with 2 mL 86% (v/v) ethanol containing 4% (v/v) acetic acid, and radioactivity in the eluate determined using a gamma counter.

Recovery of 1 μCi (1.6 ng) of ^{125}I-ANF added to a 1 mL plasma was 71 ± 1.8% (N = 6). Figures given in the text are uncorrected for recovery.

Disappearance of immunoreactive ANF in anaesthetized rats. Under pentobarbitone anaesthesia (60 mg/kg i.p.) cannulae were placed in the trachea and jugular vein of male Sprague–Dawley rats (350 g). Both kidneys were exposed via a mid-line incision, and bilateral nephrectomy or sham operation carried out. After a 10 min stabilization period, (±)candoxatrilat (3 mg/kg) or saline vehicle was injected intravenously, followed 10 min later by a 200 ng (571 ng/kg) bolus of ANF 5-28 (Atriopeptin III, Cambridge Research Biochemicals, U.K.). All injections were made in a total volume of 100 μL. Animals were killed 30, 60, 90, 120, 180 or 600 sec after ANF 5-28 injection and 10 mL blood removed via cardiac puncture into syringes