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Methods: Cell Culture and Animal Models

Materials

Sunitinib capsules were purchased from a pharmacy. For *in vitro* studies, contents of capsules were solubilized in distilled water and insoluble material was removed by repeated centrifugation at 2500g.

Caspase-9 Activity Assay

Fluorometric caspase-9 activity kits (Calbiochem) were used according to the manufacturer's instructions. Neonatal rat ventricular myocytes (NRVMs) were maintained in serum-free media overnight, and then were incubated in sunitinib or vehicle (DMSO) at the concentrations indicated in the figure for 40 hr. Caspase-9 activity is expressed, corrected for total myocyte number.

Immunocytochemistry and TUNEL Staining

NRVMs were plated on laminin-coated glass coverslips (40,000 cells/cm^2). Following treatment with sunitinib or vehicle for the times noted in figures and/or legends, cells were fixed with 4% paraformaldehyde, blocked in PBS containing 2% BSA and 0·2% horse serum, and stained with anti-cytochrome c antibody at a dilution of 1:400. Subsequently cells were washed twice and an anti-mouse FITC-conjugated secondary antibody was added at a dilution of 1:400. For visualization of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), a kit from Chemicon International was used. A Nikon Eclipse 80i microscope and software from Open Lab were used to view and record images.

Animal Studies

Animal protocols were approved by the Institutional Animal Care and Use Committees at the respective institutions (Thomas Jefferson University, Children's Hospital Boston). Age-matched wild-type Swiss-Webster mice were treated with sunitinib mixed in chow at the doses and for the times noted in the text. Osmotic minipumps (Alzet model 2002, Alza Pharmaceuticals, Palo Alto, CA) placed in the subcutaneous tissues of the back were used for administration of vehicle (0·9% saline) or phenylephrine (30 mg/kg/d). In other studies, age-matched male C57BL/6J mice (Jackson Laboratories) were treated with sunitinib (40 mg/kg/d) or vehicle via gavage for 12 days.

Blood pressure measurements - Mice were anesthetized with ketamine (50 mg/kg) and xylazine (2·5 mg/kg). A fluid-filled catheter (DSI Instruments) was inserted into the left carotid artery and the transducer with battery was placed in the subcutaneous layer of the subscapular region. Mice were allowed to recover. Blood pressure was measured via telemetry in conscious, unrestrained animals on two successive days. Measurements were made in all mice at approximately the same time of day to minimize diurnal variations in blood pressure.

Cardiac Tissue Analysis

For determination of apoptosis in mouse hearts, we employed TUNEL staining in sections of hearts using a kit from Chemicon International, exactly as described by the manufacturer.

For transmission electron microscopy (TEM) studies, 1 mm^3 pieces of the LV were cut from excised hearts and prepared for TEM following established procedures.[1] Specimens were examined using a JEOL 2100 TEM at 200 kV. At least 100 electron micrographs were examined. To reduce the sampling problem inherent in electron microscopy, 1-μm-thick sections of Epon-embedded tissues were prepared and stained with alkaline Giemsa stain.

Neonatal rat ventricular myocyte (NRVM) culture

Cultured ventricular myocytes were prepared from 2-4 day old Sprague-Dawley rats (Charles River Laboratory) as previously described.[2] For some assays, cells were cultured in F-10 medium containing 2-5% fetal bovine serum (FBS), penicillin-streptomycin (100 IU/ml), and 100 μM 5-Bromo-2′-deoxyuridine. Other assays were conducted in low glucose (LG)-DMEM, 10% FBS, 1% penicillin-streptomycin-glutamine, and 10 μg/ml cytosine-β-D-arabinofuranoside.

Statistical Analysis

All statistical analyses employed Student *t*-test for unpaired data except for Figure 5D, for which one–way analysis of variance and Bonferroni's post-test correction were used. Data are presented as mean ± s.e.m. except where noted.

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Table 1. Clinical Characteristics of Patients with Congestive Heart Failure

Age	**M/F**	**Body Weight /BMI** *(kg; BMI)*	**Starting Dose** *(mg; wks on drug/wks off drug)*	**Dose at event** *(mg; wks on drug/wks off drug)*	**Cardiac drugs at event**	**No. of wks until CHF**	**Past Cardiac History and Clinical Synposis**	**LVEF Δ (EF%)**	**Outcome**
37	F	56·2; 17·9	50; 2/2	50; 4 /2	–	71·3	No prior cardiac history. Chest tightness, SOB, elevated JVP, fatigue and bipedal edema. No BNP available. TnI nl. No CAD on cath. Biopsy done.	57 > 38	• Sunitinib withheld for 14 days. • LVEF improved to 54% in 6 weeks. • Resumed drug on 50 mg; 4 wks on/2 wks off dosing schedule. LVEF intermittently dropped to 38% when on-drug and improved off-drug. • Patient was transferred after 86·7 weeks to the continuation study.
72	M	65·5; 24·1	75; 2/2 (1 cycle only)	50; 4/2	Lisinopril, Metoprolol, HCTZ, ASA, Glyburide	84·9	H/O CAD; S/P MI 8 yrs ago. H/O HTN and DM. Intermittent LVEF declines with PND, orthopnea and CP. HTN on drug. No BNP available. TnI nl. Cath deferred secondary to renal dysfunction. Biopsy done.	60 > 34	• Sunitinib discontinued. • LVEF improved to 55% in one week. • Patient was taken off-study after 85·9 weeks due to disease progression and cardiac intolerance to sunitinib.
67	M	82·9; 28·7	50; 2/2	50; 2/2	Metoprolol, ASA	10·7	H/O CAD; S/P MI 10 yrs ago. H/O HTN, and prior anthracycline therapy. SOB, pedal edema, and ascites. BNP=290. TnI nl. Elevated R and L heart filling pressures on cath without new CAD.	52 > 36	• Sunitinib withheld for 9 days. • Treated with Spironolactone, Captopril, and Lasix. LVEF improved to 42% in 17 days. • Resumed drug on 50 mg; 2 wks on/2 wks off dosing schedule. • Patient was taken off-study after 16·7 weeks due to tumor progression/patient death.

76	F	80·6; 26·4	50; 4/2	50; 4/2	Amlodipine, Lisinopril, Atenolol	45·1	No prior cardiac history. H/O HTN. SOB at rest, fatigue, and bipedal edema. HTN on drug. CXR demonstrated bilateral pulmonary edema. BNP=1700. TnI=0.12	66 > 47	• Sunitinib withheld for 27 days. • Treated with Lasix, increased Lisinopril and transfusion for anemia. LVEF improved from 47% to 66% in 27 days. Symptoms resolved. • Resumed drug on 50 mg; 4 wks on/2 wks off dosing schedule. LVEF intermittently decreased to 50% accompanied by edema. • Patient was taken off study after 59 weeks and enrolled in the continuation study.
58	M	83·2; 27·2	50; 4/2	50; 4/2	–	21·6	No prior cardiac history. Decreased exercise tolerance. Elevated R heart pressures. No BNP available. TnI nl.	50 > 38	• Sunitinib withheld for 15 days. • Treated with Lisinopril and HCTZ. LVEF improved from 38% to 50% in 7 days, and fatigue resolved. • Resumed drug on 50 mg; 4 wks on/2 wks off dosing schedule. • Patient transferred after 37·6 weeks and enrolled in the continuation study.
48	F	43·2; 16·8	50; 4/2	50; 4/2	–	14·7	No prior cardiac history. DOE and SOB at rest. BNP=723. TnI nl. Cardiac cath was negative for CAD.	69 > 22	• Sunitinib withheld for 54 days. • Treated with Lisinopril and Atenolol. LVEF improved from 22% to 65% in 9·6 weeks. • Resumed drug at lower dose and reduced dosing period (25 mg; 2 wks on/2 wks off alternating with 50 mg; 2 wks on/2 wks off). LVEF intermittently decreased to 50% with BNP elevation. Symptoms resolved. • Patient transferred after 36.3 weeks to the continuation study.

H/O = history of, CAD = coronary artery disease, S/P = status post, MI = myocardial infarction, HTN = hypertension, DM = diabetes mellitus, PND = paroxysmal nocturnal dyspnea, CP = chest pain, nl = normal, DOE = dyspnea on exertion, ECG = electrocardiogram, ASA = aspirin, LVEF = left ventricular

ejection fraction, SOB = shortness of breath, HCTZ = hydrochlorothiazide, post-op = post-operative, JVP = jugular venous pressure, cath = catheterization, L = left, R = right, CXR = chest x-ray, BNP = brain natriuetic peptide, TnI = troponin.

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Table 2. Characteristics of Patients Treated at the FDA-approved Dose

Characteristic (n=36)		P Value[1]
	number of patients (percent)	
Male	22 (61)	0·52
Mean age - years	56·1±10·1	0·38
ECOG* performance status		
0	23 (64)	0·54
1	13 (36)	0·54
2	0 (0)	0·54
Sunitinib Treatment		
Median weeks on-study	33.5	0·26
Range (weeks)	5·0-73·9	
Dosage – 50 mg; 4 wks on/2 wks off	36 (100)	0·83
Prior Cardiac History		
CAD	0 (0)	0·30
CHF	0 (0)	1·00
Prior Cardiac Risk Factors		
HTN	10 (28)	0·99
Smoking	8 (22)	0·82
Diabetes	5 (14)	0·75
Hyperlipidemia	4 (11)	1·00
Cardiac Medications at Baseline		
ACE inhibitor	4 (11)	0·73
Beta blocker	2 (6)	0·33
Statin	2 (6)	0·99
Prior Thyroid Disease		
Hypothyroidism†	7 (19)	0·39
Prior Chemotherapy		
Anthracycline	5 (14)	0·60
Imatinib	36 (100)	1·00

ECOG = Eastern Cooperative Oncology Group; 0 = Fully active, able to carry on all pre-disease performance without restriction, 1= Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, 2 = Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours.

† History of hypothyroidism or elevated TSH at baseline.

[1] P values as compared with entire cohort (Table 1 in text).

References

1. Login GR, Galli SJ, Morgan E, Arizono N, Schwartz LB, Dvorak AM. Rapid microwave fixation of rat mast cells. I. Localization of granule chymase with an ultrastructural postembedding immunogold technique. *Lab Invest* 1987;**57**(5)**:**592–9.

2. Kerkelä R, Grazette L, Yacobi R, Iliescu C, Patten R, Beahm C, Walters B, Shevtsov S, Pesant S, Clubb FJ, Rosenzweig A, Salomon RN, Van Etten RA, Alroy J, Durand JB, Force T. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat Med* 2006;**12**(8)**:**908–16.