Supplementary methods

Cell culture

HeLa cells were purchased from the Washington University Tissue Culture Support Center and cultured in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 × non-essential amino acids (NEAA), and 1 × penicillin/streptomycin solution. HeLa cells were maintained in a 5% CO_2/95% humidified air atmosphere at 37°C.

In vitro caspase inhibition studies with ICMT-18

Enzyme inhibition assays were performed as previously reported (1). Briefly, recombinant human caspases (3, 6, and 8) and their peptide-specific substrates (Ac-DEVD-AMC, Ac-VEID-AMC, and Ac-IETD-AMC, respectively) were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of caspase-1 and caspase-7 which were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Peptide specific substrates for caspase-1 and -7 (Ac-YVAD-AMC and Ac-DEVD-AMC) were also acquired from Sigma-Aldrich. The enzymatic activity of caspases was determined by measuring the accumulation of the fluorogenic product 7-amino-4-methylcoumarin (AMC). All assays were prepared in 96-well format at a volume of 210 µl per well and consisted of: 100 mM Na+ HEPES (pH 7.4), 10% sucrose, 100 mMNaCl, 0.1% CHAPS, 5 mM 2-mercaptoethanol, 2 mM EDTA, 10 µM Ac-YVAD-AMC (caspase-1); 20 mM Na+ HEPES (pH 7.4), 10% sucrose, 100 mMNaCl, 0.1% CHAPS, 2 mM EDTA, 10 µM Ac-DEVD-AMC (caspase-3); 20 mM Na+ HEPES (pH 7.4), 10% sucrose, 100 mM NaCl , 0.1% CHAPS, 2 mM EDTA, 10 µM Ac-VEID-AMC (caspase-6); 20 mM Na+ HEPES (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 5 mM 2-mercaptoethanol, 2 mM EDTA, 10 µM Ac-DEVD-AMC (caspase-7); 20 mM Na+ HEPES (pH 7.4), 10% sucrose, 100 mM NaCl, 0.1% CHAPS, 2 mM EDTA, 10 µM Ac-IETD-AMC (caspase-8).

Recombinant caspases were first assayed to determine the optimal concentration for each experiment. Optimal concentrations were based in the linear range of the enzyme activation curves. Peptide inhibitors with known IC_{50} values were tested together with the compounds as a control for each caspase assay.Peptide inhibitors, Ac-DEVD-CHO (caspase-3 and -7), Ac-VEID-CHO (caspase-6), and Ac-IETD-CHO (caspase-8) were purchased from Sigma-Aldrich (St. Louis, MO) with exception of caspase-1 specific inhibitor (Ac-YVAD-CHO) which was acquired from BIOMOL Research Laboratories (Plymouth Meeting, PA). Peptide inhibitors and ICMT-18 were dissolved in DMSO and a serial dilution was performed prior to screening in order to obtain desired concentrations. 10 µl was added to each well containing 100 µl caspase solution and allowed to incubate on ice for 30 minutes. 100 µl substrate solution was added to each well and plates were incubated for 1-2 hours at 37°C. The final concentration of DMSO in all wells was 5% of the total volume.

The amount of AMC released was determined by using a Victor3 microplate fluorometer (Perkin Elmer Life Sciences, Boston, MA) at excitation and emission wavelengths 355 nm and 460 nm, respectively. Compounds were tested in duplicate and IC_{50} curves were calculated for all inhibitors assayed as previously described [1]. Final IC_{50}'s were the average of 3 independent experiments. The results are depicted in Supplemental Figure 3, Supplementary Materials.

In vitro cold competition cell uptake studies with [^{18}F]WC-4-116

HeLa cells were plated 2 × 10^5 cells per well in 12-well plates 24 hours prior to the cell uptake study, ensuring 70% confluency of the cells and complete adherence to the plate. Six hours before the initiation of the experiment, 2 µM of staurosporine was added to each well to induce apoptosis and caspase-3 activation. For cold competition studies, 5 µM unlabeled WC-4-116 was first added to selected wells prior to the administration of [^{18}F]WC-4-116. At time zero, 1 µCi [^{18}F]WC-4-116 (specific activity, 5480 mCi/µMol) was added to each well. After incubation of 5, 15, and 30 min the culture medium was removed and transferred into 2 ml microcentrifuge tubes for scintigraphic analysis. The cells were then washed once with 1 ml of cold phosphate buffered saline; the wash then collected for scintigraphy as well. The cells were lysed in the 12-well plates by treatment with 0.2% sodium dodecyl sulfate solution.

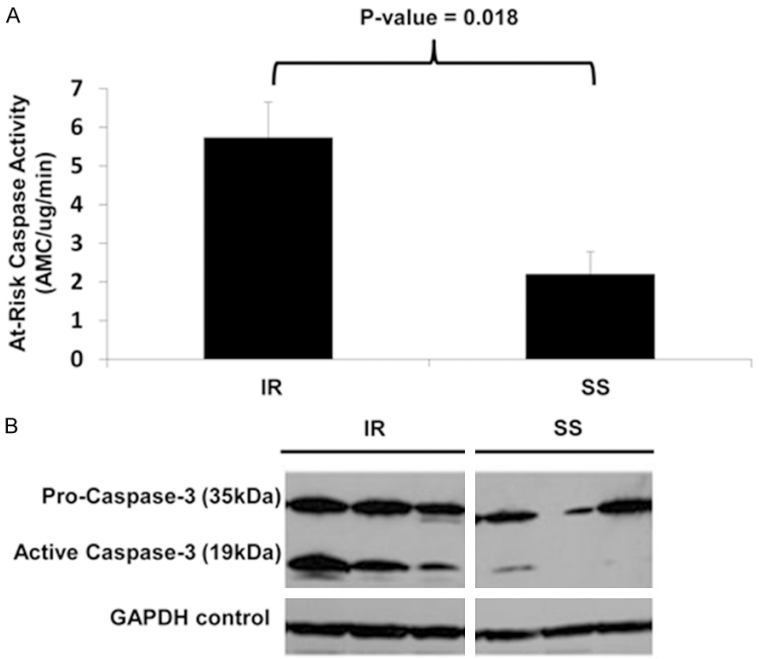
The cell lysates were harvested and the radioactivity content of the culture medium, wash, and cellular lysate were measured using a 1480 Wizard2 gamma counter (Perkin Elmer, Turku, Finland). The assay was performed in triplicate and results are reported as counts per min (CPM) of activity in the cell lysates for the samples with and without excess (unlabelled) WC-4-116 and depicted in Supplemental Figure 4, Supplementary Materials.

Quantitation of at-risk myocardial blood flow of IR and SS animals

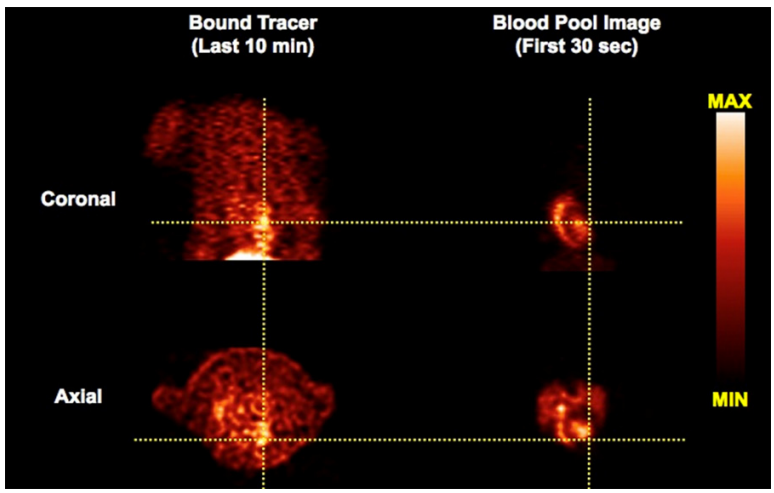
See microPET imaging protocol section in Methods for details. Mean myocardial blood flow ± standard errorof the at-risk left ventricular region was assessed by adjunctive [^{15}O]H_2O microPET imaging for the IR and SS animals prior to the administration of either [^{18}F]WC-4-116 or [^{18}F]ICMT-18. All treatments are n = 4 except for the SS group imaged with [^{18}F]WC-4-116 (n = 3), with a *p*-value (ANOVA) = 0.29 for interaction between radiotracer and treatment type. The results are depicted in Supplemental Figure 6, Supplementary Materials.

Reference

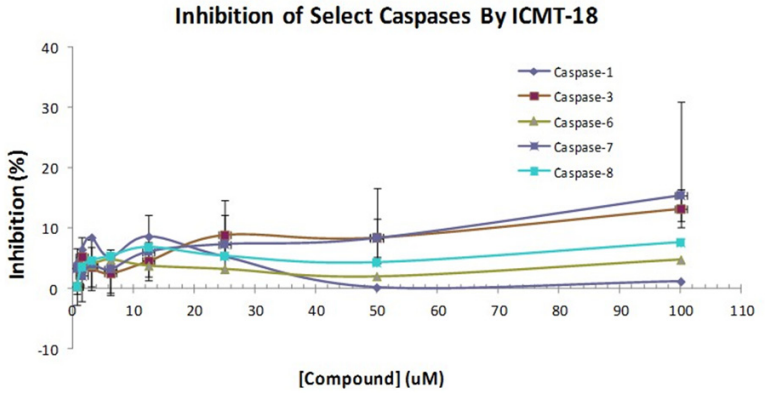
[1] Chu W, Rothfuss J, Chu Y, Zhou D, Mach RH. Synthesis and in vitro evaluation of sulfonamide isatin Michael acceptors as small molecule inhibitors of caspase-6. J Med Chem. Apr 23 2009; 52: 2188-2191.



Supplement Figure 1. The *in vivo* LAD ligation model produces increased caspase-3 activity and expression. Panel A. Mean caspase-3/7 activity (± standard error) is elevated in the at-risk myocardium of the IR animals (n = 5) compared with the SS animals (n = 3). B. Western immunoblotting demonstrates enhanced expression of the active caspase-3 19 kDa fragment in the at-risk myocardial tissue samples for the IR but not the SS animals. Expression of the 35 kDA pro-caspase fragment and GADPH loading control are similar between treatments. AMC and GADPH denote amido-4-methylcoumarin and glyceraldehyde 3-phosphate dehydrogenase respectively.



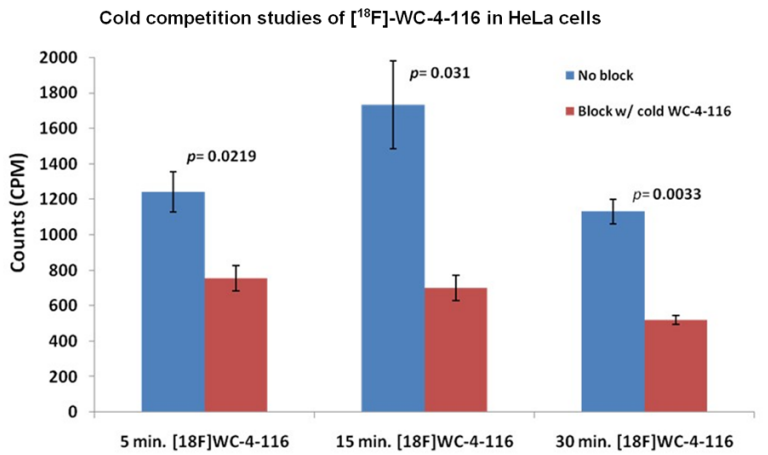
Supplemental Figure 2. Summed images late (left) and early (right) to differentiate tracer uptake in the myocardium and in the blood pool.



Inhibition of select caspases by ICMT-18

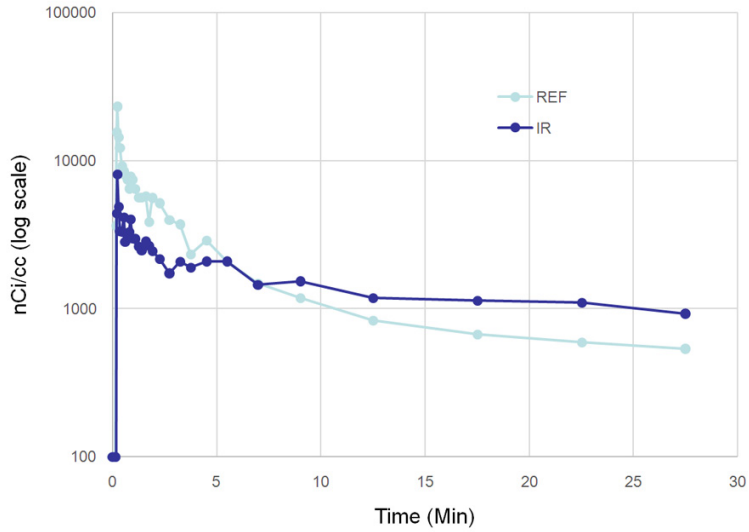
Caspase	Inhibition @ 100μM (%)
Caspase-1	2.3 ± 0.9
Caspase-3	15.3 ± 2.7
Caspase-6	5.8 ± 2.1
Caspase-7	18.4 ± 4.4
Caspase-8	7.8 ± 1.5

Supplemental Figure 3. Inhibition studies of caspases 1, 3, 6, 7, and 8 with ICMT-18. As expected, ICMT displayed no inhibition of caspase activity at a concentration up to 100 mM.

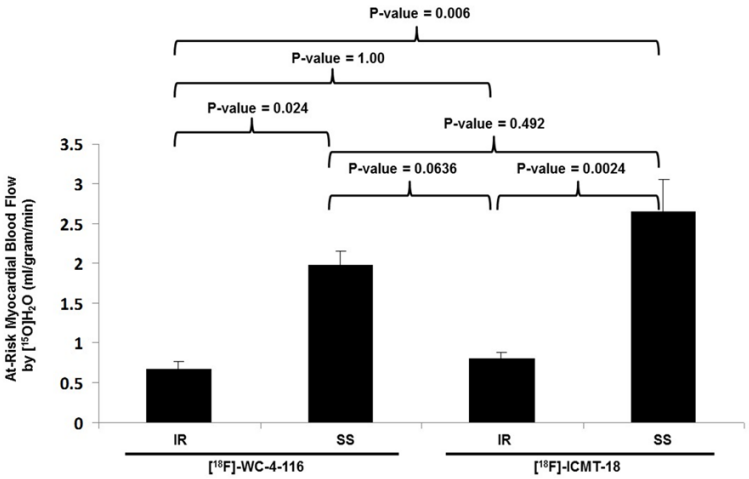


Uptake of compound measured in counts (CPM)		
Incubation Time	No Block	Block w/WC-4-116
5 min. [^{18}F]WC-4-116	1242 ± 112	758 ± 72
15 min. [^{18}F]WC-4-116	1734 ± 246	702 ± 74
30 min. [^{18}F]WC-4-116	1132 ± 70	521 ± 26

Supplemental Figure 4. Blocking studies in staurosporine-treated HeLa cells. Addition of unlabeled WC-4-116 is consistent with carrier-added blocking of caspase-3.



Supplemental Figure 5. Log-scale of TAC to highlight 2-fold ratio between IR and reference region at late time points.



Supplemental Figure 6. [^{15}O]H_2O blood flow studies in IR and sham surgery controls. Although the IR animals had lower blood flow in the region at risk relative to the sham surgery controls, there was no difference in blood flow between the animals receiving [^{18}F]WC-4-116 versus [^{18}F]ICMT-18 (*P* value = 1.00). These data confirm that the increase uptake of [^{18}F]WC-4-116 versus [^{18}F]ICMT in the region at risk in the IR animals is due to binding to activated caspase-3 and not due to differences in myocardial blood flow.