Novel antagonists of the thioesterase domain of human fatty acid synthase

Robyn D. Richardson and Jeffrey W. Smith
Cancer Research Center and Center on Proteolytic Pathways, Burnham Institute for Medical Research, La Jolla, California

Abstract
Fatty acid synthase (FAS) is up-regulated in a wide range of cancers and has been recently identified as a potential therapeutic target. Indeed, previous research has shown that inhibition of FAS with active site-modifying agents can block tumor cell proliferation, elicit tumor cell death, and prevent tumor growth in animal models. Here, we use a high-throughput fluorogenic screen and identify a novel pharmacophore, 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione, which inhibits the thioesterase domain of FAS. The novel antagonists are competitive inhibitors of the thioesterase domain, inhibit de novo fatty acid synthesis, and elicit FAS-dependent tumor cell death. This set of novel FAS antagonists provides an important pharmacologic lead for further development of anticancer therapeutics. [Mol Cancer Ther 2007;6(7):2120–6]

Introduction
Fatty acid synthase (FAS) is the sole enzyme in mammals responsible for cellular synthesis of palmitate, the precursor for most fatty acids. FAS has six separate enzymatic pockets that act sequentially to condense acetyl CoA and malonyl CoA, ultimately generating palmitate linked to the acyl carrier protein. Palmitate is liberated from the enzyme by the COOH-terminal thioesterase domain via a serine hydrolase mechanism. FAS activity has little effect in normal cells because they take up and use dietary lipids. However, tumor cells behave differently. FAS is up-regulated in various tumor types, including those of the breast (1–3), prostate (4–6), and ovaries (7–9), and it has been linked to poor prognosis in these cancers. This linkage between normal and tumor cells could lead to de novo synthesis of fatty acids. This difference in FAS dependence between normal and tumor cells could lead to a favorable therapeutic index for drugs that target FAS.

Covalent inhibitors of FAS, such as orlistat (Xenical®), c75, and cerulenin, block tumor cell proliferation in vitro and decrease the growth of tumor xenografts in mice (10, 11). Equally as important, recent evidence shows that the pharmacologic inhibition of FAS sensitizes tumor cells to chemotherapeutic agents, such as Taxol (12) and Herceptin (13). Unfortunately, cerulenin and c75 are known to have significant affinity for other protein targets (14, 15), and orlistat is poorly soluble. Consequently, these compounds are not ideal for deployment as anticancer drugs. Given that FAS holds so much promise as a druggable target and the paucity of suitable inhibitors, we sought to identify an alternative class of FAS antagonists that are suitable leads for drug design.

Here, we report on the identification of new classes of antagonists of the thioesterase domain of FAS. More than 37,000 compounds were screened to identify a 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione–based pharmacophore that inhibits the FAS thioesterase. The most potent of these compounds blocks the activity of the FAS holoenzyme and is cytotoxic to the lipogenic MB-MDA-435 breast cancer cells but much less toxic to the nonlipogenic breast epithelial cells (16, 17). A comprehensive search of public databases and literature revealed no known connection between the pharmacophore and FAS (or any other serine hydrolase). Therefore, these compounds represent a novel class of serine hydrolase inhibitors.

Materials and Methods
Expression and Purification of the FAS Thioesterase
Expression of the recombinant thioesterase domain of FAS using pTrcHis-TOPO vector (Invitrogen Corp.) was described previously (10). Large-scale expression and purification was done by Invitrogen.

Biochemical Compound Screening
The primary screen of 36,500 compounds from the DIVERSet Collection (ChemBridge) and the secondary screen of 515 structurally related compounds (ChemBridge) were done in 96-well Fluorotrac 200 plates (Greiner) using 4-methylumbelliferyl heptanoate (4-MUH; Sigma) as a fluorogenic substrate (18, 19). The optimal substrate concentration was 120 μmol/L 4-MUH or approximately 5 × K_m. Briefly, reaction mixtures contained FAS thioesterase in buffer A [45 μL; 100 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.05% Brij 35] or buffer A alone. Controls included protein solution plus vehicle (DMSO) to determine untreated enzyme activity and buffer A plus DMSO to quantify background hydrolysis of the fluorogenic substrate. Compounds (5 μL) or a 10% (v/v) DMSO solution (control) was added to yield final concentrations of approximately 12 μmol/L (for primary screen) and 1 μmol/L (for secondary screen). The plates were incubated...

Received 3/16/07; revised 5/3/07; accepted 5/25/07.
Grant support: NIH grant AI055789 (R. Liddington), CA108959 (J.W. Smith), AI061139 (A. Strongin), and CA030199 (K. Vuori).
Requests for reprints: Jeffrey W. Smith, Cancer Research Center and Center on Proteolytic Pathways, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037. Phone: 858-646-3100; Fax: 858-713-9921. E-mail: jsmith@burnham.org
Copyright © 2007 American Association for Cancer Research. doi:10.1158/1535-7163.MCT-07-0187

1 Recent studies have generated questions about the origin of this cell line.

at 37°C for 30 min before adding 4-MUH in 5 μL DMSO/buffer A (1:1). Final DMSO concentration was 5.5% (v/v). Plates were incubated at 37°C for 60 min and assayed at 360/435 nm. Compounds that inhibited enzymatic activity >40% (for primary screen) or >50% (for secondary screen) were examined further. Compounds that were examined in great detail required the purchase of additional dry material and were assayed for identity to that in the parent library and the reported structure by liquid chromatography-mass spectrometry (data not shown).

Concentration-Response Curves to Determine IC₅₀ Values
To establish the potency of each compound, they were tested at concentrations 0.16 to 10 μmol/L. Data points were collected in triplicate and each experiment was done at least twice independently. Reaction volumes contained 2 μL of each dilution of lead antagonist or vehicle (DMSO) with 45 μL of 500 nmol/L FAS thioesterase in buffer A or buffer A alone. Plates were preincubated for 30 min at 37°C before adding 5 μL 120 μmol/L 4-MUH in 1:1 DMSO/buffer A. Final DMSO concentration was 8.7% (v/v). Fluorescence was monitored every 5 min for 40 to 60 min to generate concentration-response curves from which IC₅₀ values were determined. The inhibition constants (Kᵢ) were calculated using the equation of Cheng and Prusoff (20).

Kinetic Characterization of Inhibitors
To characterize the mechanism of action of selected compounds, their ability to block the turnover of 4-MUH (10–60 μmol/L) was measured in the presence of 500 nmol/L FAS thioesterase across a concentration range. The reaction mixtures contained 2 μL lead antagonist or vehicle (DMSO) with 45 μL of 500 nmol/L FAS thioesterase in buffer A or buffer A alone. The final DMSO was 8.7% (v/v). Plates were preincubated for 30 min at 37°C before adding 10 to 60 μmol/L 4-MUH in DMSO/buffer A (1:1). The formation of fluorescent product was monitored in 5-min intervals for 40 to 60 min. Linear regression curves were analyzed using GraphPad Prism (Biosoft).

Cell Culture
MDA-MB-435 breast cancer cells (11, 21) were used for biological testing of the lead antagonists. MDA-MB-435 cells undergo cell cycle arrest and apoptosis when FAS is inhibited, thereby providing a model platform. Cells were maintained in minimal Eagle’s medium, Earle’s salts (Irvine Scientific) supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mmol/L L-glutamine (Invitrogen), minimal Eagle’s medium vitamins (Invitrogen), nonessential amino acids (Irvine Scientific), and antibiotics (Omega Scientific) at 37°C and 5% CO₂. The MCF-10A cell line is a well-characterized nonlipogenic cell line and was therefore used as a model for normal levels of FAS dependence (17, 22). Cells were maintained in the complete medium described above supplemented with 100 ng/mL recombinant human epidermal growth factor (Sigma) at 37°C and 5% CO₂. MDA-MB-435 cells were generously provided by Dr. Janet Price (University of Texas, Austin, TX). All other cell lines were purchased from the American Type Culture Collection.

Measuring Fatty Acid Synthesis by the FAS Holoenzyme
Fatty acid synthesis by the FAS holoenzyme in cell lysates was measured by incorporation of [¹⁴C]malonyl-CoA (Amersham). MDA-MB-435 cells (5 × 10⁶ total) were lysed by sonication in buffer B [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT]. Each reaction contained 20 μg total cellular protein and 5 or 20 μmol/L of inhibitor or vehicle as a control. The final DMSO concentration was 1.5% (v/v). Samples were incubated on ice for 30 min before addition of reaction mixture (130 μL; 115 mmol/L KCl, 192.2 μmol/L acetyl-CoA, 576.9 μmol/L NADPH) and [¹⁴C]malonyl-CoA (5 μL; 0.1 μCi). Samples were incubated at room temperature for 15 min and fatty acids were extracted with chloroform/methanol (1:1). The chloroform fractions were dried and reextracted with hydrated butanol/water (1:1). The butanol fractions were reduced to 300 μL and added to EcoLume (ICN Biomedicals) scintillation fluid (4 mL). Labeled fatty acids were detected by scintillation. All samples were prepared in duplicate and the experiment was done at least twice.

Figure 1. Screening strategy for antagonists of human FAS. The biochemical assay (triangles) was used to screen more than 36,500 drug-like compounds each at a concentration of 12 μmol/L. Turnover of the 4-MUH substrate by the thioesterase yields fluorescence on liberation of the 4-methylumbelliferone. Five compounds contained the maximum common structure (MCS), which is a putative pharmacophore for FAS thioesterase. A focused library of 515 compounds was purchased based on the pharmacophore. A secondary screen was done against this focused library at ~1 μmol/L. A parallel cytotoxicity screen using human foreskin fibroblasts (oval) was also done. Compounds (~195) yielding viabilities >90% tend to correlate with precipitating compounds and were dropped from the study. In addition, compounds (~100) that left <10% viable cells after treatment were dropped due to unacceptable toxicity. Forty compounds with suitable activity, solubility, and toxicity were studied in greater detail.
**Measuring Cytotoxicity**

For cytotoxicity experiments, MB-MDA-435 and MCF10-A cells were plated in 96-well plates at $3 \times 10^3$ per well in 200 μL appropriate complete MEM and incubated overnight at 37°C and 5% CO₂. Cells were treated with lead antagonists (0.78–100 μmol/L) or vehicle in triplicate, with a final percentage of DMSO not exceeding 1% (v/v). After 48 h, the medium was aspirated and replaced with complete MEM containing 333 μg/mL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 25 μmol/L phenazine methosulfate using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Plates were incubated for 2 h and absorbance was assayed at 490 nm. Background levels of formazan formation were measured in medium alone. IC₅₀ values were derived by fitting triplicate data points over the concentration range with the “dose–response–inhibition” function of GraphPad Prism. Each experiment was done at least twice.

**Results**

**A Chemically Diverse Compound Library Contains Antagonists of the FAS Thioesterase Domain**

The activity of the recombinant thioesterase was assessed by its ability to cleave 4-MUH, which is hydrolyzed to the fluorescent 4-methylumbelliferone (18, 19). We initially screened a library of 36,500 drug-like compounds. The primary screen was conducted at a concentration of ~12 μmol/L of each compound, revealing 116 compounds that blocked >40% of the thioesterase activity (Fig. 1). These compounds were retested to confirm activity. Eighteen compounds were identified with an apparent $K_i$ of <1.0 μmol/L, 5 of which contain the

<table>
<thead>
<tr>
<th>Structural element</th>
<th>Frequency ¹</th>
<th>Active (%) ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_1 =$ Br or I</td>
<td>35</td>
<td>94</td>
</tr>
<tr>
<td>S*</td>
<td>17</td>
<td>88</td>
</tr>
<tr>
<td>CH₃</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>H</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>NO₂</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>N(CH₃)₂ or N(CH₂CH₃)₂</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>O</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>N*</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>N*</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Although the carbon backbone is drawn here for clarity, the individual E/Z configurations have not been determined.

¹Indicates point of connectivity.

¹Active is defined as having ≥50% inhibition at 1 μmol/L.

¹Substitutions with frequency <10 are excluded.
A parallel screen was done to determine general toxicity of the compounds on human foreskin fibroblasts at a concentration of 100 μmol/L (data not shown). Compounds (~195) yielding viabilities >90% tend to correlate with precipitating compounds and were dropped from the study. In addition, compounds (~100) that left <10% viable cells after treatment were dropped due to unacceptable toxicity. The compounds with suitable activity against the FAS thioesterase and minimal toxicity were then analyzed.

5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione–based pharmacophore. This pharmacophore had not previously been reported as a serine hydrolase inhibitor and represents the basis for promising lead compounds as inhibitors of FAS.

Structure-Activity Trends Emerge from Studies with the Focused Library

A focused library containing 515 derivatives of the 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione pharmacophore was characterized. All of the compounds contain this maximum common substructure, with varied substitutions at R1 and either at a single or at both amines in the pyrimidine ring. A secondary screen against this focused library was conducted at a concentration of ~1 μmol/L of each compound using the fluorogenic assay described above. Under these conditions, just over half (286) of the compounds inhibited FAS thioesterase activity by at least 50% (Fig. 1). Interestingly, substitutions at R1, h to the furan ring, had the most dramatic effect on activity (Table 1). Generally, substitutions at this site with high electron density, such as bromine, iodine, or phenyl rings, exhibit potent activity. In contrast, substitutions with relatively low electron density, such as a methyl group and alkyl amines (both cyclic and acyclic), or no substitution at all, show little or no activity despite substitutions at other sites. Substitutions at R2, those at an amine on the pyrimidine ring, seem to make less of a contribution to binding because a range of chemically diverse substitutions fails to affect activity. This suggests that this portion of the compound does not interact directly with the protein. However, a bulky disubstituted pyrimidine ring has deleterious effects on binding because, of the 42 compounds in the library that are substituted at both amines, only 6 are active; all of them are N-N’-dimethyl. This may indicate steric hindrance with the positioning of the inhibitor into its binding pocket.

A parallel screen was done to determine general toxicity of the compounds on human foreskin fibroblasts at a concentration of 100 μmol/L (data not shown). Compounds (~195) yielding viabilities >90% tend to correlate with precipitating compounds and were dropped from the study. In addition, compounds (~100) that left <10% viable cells after treatment were dropped due to unacceptable toxicity. The compounds with suitable activity against the FAS thioesterase and minimal toxicity were then analyzed.
The Lead Antagonists Are Potent, Competitive Inhibitors of the FAS Thioesterase Domain

Kinetic analysis was used to determine the \( K_i \) value for selected lead antagonists and to assess the general mechanism of their inhibition of the FAS thioesterase domain. \( K_i \) values were determined by concentration-response curves and analyzed with the equation of Cheng and Prusoff (20) and/or by Lineweaver-Burke plots. The representative double reciprocal plots of the \( V_I \) and 4-MUH data revealed that compounds from both class A and B are competitive inhibitors as indicated by linear regression plot lines intersecting at \( 1/V_{max} \) for the untreated controls (Fig. 3A and B). The corresponding data for orlistat, a known FAS inhibitor, are included for comparison. These analyses were done using GraphPad Prism software. As expected from the screening data, the \( K_i \) values are submicromolar in this assay and are comparable with that of orlistat.

The Lead Antagonists Inhibit the FAS Holoenzyme

To test inhibitory activity against full-length FAS, we measured the effect of the compounds on de novo fatty acid synthesis in cell lysates, where the FAS holoenzyme remains active. The incorporation of \([14C]\) malonyl CoA, a precursor of palmitate, into fatty acids was measured according to published methods (23). Treatment of cell lysates with 20 \( \mu \)mol/L of selected compounds abrogated fatty acid biosynthesis by \( >90\% \) for the most potent compounds within 15 min (Fig. 4). Treatment with 5 \( \mu \)mol/L of the same compounds inhibited de novo fatty acid synthesis an average of 52\%. These numbers roughly correlate with the corresponding cytotoxicity IC50 values in MB-MDA-435 cells and confirm activity against the FAS holoenzyme. Interestingly, compound 4, a congener of cyclic system B, showed no significant difference in the amount of inhibition when the 5 \( \mu \)mol/L and the 20 \( \mu \)mol/L concentrations were compared.

The Lead Antagonists of FAS Are More Cytotoxic to MDA-MB-435 Cancer Cells than to Nonlipogenic Cells

Lead antagonists were tested for their ability to kill MDA-MB-435 cells, which are FAS dependent, or MCF-10A cells, which are immortal but not highly lipogenic (17, 22). Concentration-response curves of cell viability were generated for selected compounds to calculate IC50 values (Table 2). The IC50 values for the MD-MBA 435 cells are generally 2- to 5-fold lower than those observed for the MCF-10A cells and roughly correspond to the concentrations required for 50\% inhibition of fatty acid biosynthesis (Fig. 4). A few lead antagonists (such as compound 4; Table 2) exhibited no apparent toxicity toward MCF-10A cells at concentrations up to 50 \( \mu \)mol/L. The data for the known FAS inhibitor, orlistat, are in good agreement with that of the lead antagonists; orlistat has a 3-fold lower IC50 in the MD-MBA-435 cells than in the MCF-10A cells. Altogether, these observations are generally consistent with the idea that the cytotoxic effects of the compounds are a result of the inhibition of FAS in whole cells. Nevertheless, the possibility that the compounds react with additional cellular targets cannot be excluded.

Discussion

The objective of this study was to identify novel antagonists of the thioesterase domain of human FAS that might be useful for further drug development. We screened more than 36,500 drug-like compounds and identified a 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione–based pharmacophore. Derivatives of this pharmacophore (a) are competitive inhibitors of the recombinant thioesterase, (b) inhibit the FAS holoenzyme and block fatty acid synthesis, and (c) elicit breast cancer cell death at concentrations significantly lower than those toxic to nonlipogenic breast epithelial cells. Based on these observations, these compounds represent a unique class of FAS antagonists that could be further developed as antineoplastic agents.

The compounds described here fulfill the Lipinski rule-of-five analysis, a guideline used to identify drug-like molecules for preclinical development (24). Lead compounds with high cLogP values are less likely to be successful drug candidates due to poor systemic absorption and membrane permeability. The FAS inhibitor orlistat (cLogP = 8.609), for example, is highly insoluble under physiologic conditions, with current use limited to the gut (25). The compounds presented in this study have cLogP \( \geq \) 5, suggesting the potential for both aqueous solubility and suitable membrane permeability. Therefore, we believe that...

---

\( ^2 \) http://www.pannanugget.com
the identified compounds represent a promising pharmacophore for development of drugs targeting FAS.

Both the initial screen for FAS thioesterase antagonists and the subsequent kinetic characterizations were done using 4-MUH as a mimic of the natural substrate. We found that the lead antagonists inhibited 4-MUH turnover by thioesterase and also blocked fatty acid synthesis by full-length FAS as measured via incorporation of a radiolabel. Therefore, the simplest interpretation of our findings is that the 4-methylumbelliferone substrate is a reasonable mimic of the natural substrate and that the lead antagonists we have identified can antagonize the thioesterase in near physiologic conditions. However, a small percentage of the lead antagonists, compound 4 in particular, showed no significant difference in FAS inhibition when the 5 μmol/L and the 20 μmol/L concentrations were compared. Future efforts to obtain cocrystal structures should provide insight into the inhibitory mechanisms of both classes (cyclic systems A and B). The position of the inhibitors in the large palmitate binding pocket and the difference in binding, if any, between the two classes will be of particular interest and will guide future synthetic efforts.

Our findings also show that the lead antagonists are competitive antagonists of the thioesterase, meaning that they bind to the unoccupied enzyme and reduce turnover of substrate. FAS is a multidomain enzyme and contains an ACP to which the elongating fatty acid alkyl chain is bound during biosynthesis. The resulting palmitoyl-ACP complex is just 48 Å from the thioesterase active site (26) where it is hydrolyzed to free palmitate. Hence, the “effective concentration” of native substrate for the thioesterase is high. Competitive inhibitors must meet a high hurdle to compete with endogenous substrate. Given the substantial potency of the lead compounds even under these challenging conditions, it is likely that optimization of these lead inhibitors will yield compounds suitable for testing in vivo.

The barbituric acid moiety found in the pharmacophore is common to pharmaceutically important compounds,

Table 2. Structures, $K_i$ values, and cytotoxicity of selected lead antagonists

<table>
<thead>
<tr>
<th>Structure</th>
<th>$K_i$ (μmol/L)</th>
<th>MB-MDA-435 IC$_{50}$ (μmol/L)</th>
<th>MCF-10A IC$_{50}$ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.38 ± 0.1</td>
<td>3.47 ± 0.8</td>
<td>24.3 ± 3.0</td>
</tr>
<tr>
<td>2</td>
<td>0.91 ± 0.2</td>
<td>4.1 ± 0.5</td>
<td>8.0 ± 2.3</td>
</tr>
<tr>
<td>3</td>
<td>0.88 ± 0.10</td>
<td>8.9 ± 3.0</td>
<td>29.0 ± 14.6</td>
</tr>
<tr>
<td>4</td>
<td>0.85 ± 0.16</td>
<td>9.5 ± 2.8</td>
<td>&gt;50</td>
</tr>
<tr>
<td>5</td>
<td>0.30 ± 0.09</td>
<td>20.0 ± 0.2</td>
<td>72.1 ± 1.6</td>
</tr>
</tbody>
</table>

NOTE: All data points were done in triplicate. IC$_{50}$ values are the mean ± SE of at least two experiments (n = 2). Individual E/Z configurations have not been determined for compounds 1 to 4. Compound 5 is orlistat, a known inhibitor of FAS thioesterase, which contains a β-lactone moiety.
such as phenobarbital and pentobarbital. Given the similarity in chemical structure between these drugs and the thioesterase antagonists, we tested phenobarbital and the core barbiturate moity for the ability to inhibit the FAS thioesterase and found both to be without effect in the fluorogenic assay at concentrations up to 100 μmol/L (data not shown). Additionally, the FAS thioesterase lacks any structural homology to the γ-aminobutyric acid–mediated chloride channel family of proteins targeted by pheno-barbital and pentobarbital (27–29). Modeling of pentobarbital binding to chloride channels reveals steric hindrance of 5'-methylbutyl side chains with amino acids protruding from the ion channel (30–32). It is tempting to speculate that the bulky ring structures at positions R1 and R2 (see Table 1) found in cyclic systems A and B would likewise inhibit binding to the targets of current clinical barbiturates, which would render central nervous system side effects negligible.

Overall, the compounds identified here are promising leads that exhibit pharmacologic advantages over previously described FAS inhibitors. These compounds block fatty acid synthesis, exhibit selective cytotoxicity against FAS-dependent breast cancer cells, and satisfy the Lipinski rule-of-five analysis. Thus, our current study builds a solid framework for further optimization and preclinical testing of compounds containing the 5-((furan-2-ylmethylene) pyrimidine-2,4,6-trione pharmacophore.

Acknowledgments

R.D. Richardson thanks Drs. Adam D. Richardson and Ziwei Huang for helpful discussions. We thank Dr. Mark Johnson for creating and making the Meqi software available to us.

References

Novel antagonists of the thioesterase domain of human fatty acid synthase

Robyn D. Richardson and Jeffrey W. Smith


Updated version  Access the most recent version of this article at:
http://mct.aacrjournals.org/content/6/7/2120

Cited articles  This article cites 32 articles, 10 of which you can access for free at:
http://mct.aacrjournals.org/content/6/7/2120.full.html#ref-list-1

Citing articles  This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/6/7/2120.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.