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Award Number:

W81XWH-08-1-0150

TITLE:

A Cholesterol-Sensitive Regulator of the Androgen Receptor

PRINCIPAL INVESTIGATOR:

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CONTRACTING ORGANIZATION:

Children's Hospital
Boston, MA 02115

REPORT DATE:

JULY 2010

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	3. DATES COVERED (FROM-TO)
01-07-2011	Annual	

4. TITLE AND SUBTITLE

A Cholesterol-Sensitive Regulator of the Androgen Receptor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0150

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital

Boston, MA 02115-5737

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRAA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

X Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Published studies from our laboratory have shown that critical mediators of tumor cell physiology and behavior reside in cholesterol-rich, lipid raft membranes of prostate cancer cells. This project focuses on the RNA binding protein heterogeneous nuclear ribonucleoprotein K (hnRNP-K) as a novel regulator of the androgen receptor (AR). We have found that hnRNP-K lies within the cholesterol-sensitive PI3K/Akt/PTEN/mTOR pathway and that hnRNP-K connects ErbB receptor/Akt-derived signals with androgenic signals, thereby directly linking peptide hormone and steroid hormone signal transduction mechanisms. Our ***hypothesis*** is that hnRNP-K mediates androgen sensitivity and growth and survival in prostate cancer cells by a mechanism involving the regulation of AR mRNA translation. The specific aims are:

Aim 1. Determine the mechanism(s) underlying the effects of hnRNP-K on androgen sensitivity in prostate cancer cells.
Aim 2. Determine whether changes in expression and/or subcellular localization alter the function of hnRNP-K and assess the physiologic consequences of these changes in prostate cancer cells.

15. SUBJECT TERMS
hnRNP-K, androgen receptor, mRNA translation, prostate cancer progression

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

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A Cholesterol-sensitive Regulator of the Androgen Receptor
Grant number: W81XWH-08-1-0150
Progress report for 07/01/2009-11/30/2010, prepared by Michael R. Freeman, PhD

INTRODUCTION

Published studies from our laboratory have shown that critical mediators of tumor cell physiology and behavior reside in cholesterol-rich, lipid raft membranes of prostate cancer cells. This project focuses on the RNA binding protein heterogeneous nuclear ribonucleoprotein K (hnRNP-K) as a novel regulator of the androgen receptor (AR). We have found that hnRNP-K lies within the cholesterol-sensitive PI3K/Akt/PTEN/mTOR pathway and that hnRNP-K connects ErbB receptor/Akt-derived signals with androgenic signals, thereby directly linking peptide hormone and steroid hormone signal transduction mechanisms. Our ***hypothesis*** is that hnRNP-K mediates androgen sensitivity and growth and survival in prostate cancer cells by a mechanism involving the regulation of AR. The specific aims are:

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BODY

As described in last year's progress report, we have made extensive progress on: *Aim 1, Task 1. Determine the mechanism of the effects of hnRNP K on AR expression. Aim 1, Task 3. Generate hnRNP K mutants that differentially regulate the AR. Aim 2, Task 1. Determine the physiological consequences of altering hnRNP K expression in prostate cancer cells.* These findings were reported in a leading journal (Mukhopadhyay et al. Cancer Res. 69:2210-2218, 2009).

In this progress report, we describe significant accomplishments from ***Aim 2, Task 3***: *Identification of hnRNP K interacting proteins in prostate cancer cells.*

Androgen receptor (AR) interacts with a variety of proteins to regulate gene expression during prostate cancer (PCa) progression. In studies funded by this grant, we have identified the nuclear matrix protein SAFB1 as a novel AR co-repressor as well as an interacting partner and phosphorylation target of the pro-survival kinase Akt1 and the growth inhibitory kinase Mst1.

SAFB1 is a nuclear matrix-associated protein that was originally identified by its ability to bind to scaffold/matrix attachment regions (S/MARs) and to the small heat shock protein hsp27 gene promoter. SAFB1 and its paralog SAFB2 contain several functional domains, including a DNA binding region (SAP/SAF-box), an RNA recognition motif, a nuclear localization signal, and Glu/Arg, Ser/Lys, and Gly-rich protein interaction domains. SAFB1 resides in the nucleus and has been linked to a variety of cellular processes, including transcription, cell cycle regulation, apoptosis, differentiation, and stress response. Several reports indicate that the primary role of SAFB1 is transcriptional repression of nuclear steroid receptors and silencing of different genes including estrogen mediated repression of genes. Recently published data also indicate that SAFB1 regulates the expression of a number of genes critical in the immune system, such as chemokines, interleukins, and members of the major histocompatability complex.

We identified SAFB1 as an interacting partner with hnRNP-K in immunoprecipitation experiments in which activated Akt1 was expressed ectopically in LNCaP prostate cancer cells. Biochemical experiments indicated that SAFB1 is a substrate for the serine-threonine kinases Mst1 and Akt1. In promoter-reporter experiments and chromatin immunoprecipitation (ChIP) experiments we implicated Mst1 directly in the ability of SAFB1 to repress transcription from AR-regulated promoters, and the ability of Mst1 to localize to these regions was dependent on SAFB1. We subsequently found that the inhibitory activity of SAFB1 involved histone H3 trimethylation on lysine 27 (H3K27), and coincided with the presence of the polycomb repressive complex 2 (PCR2), including the H3K27 trimethylase EZH2. EZH2 has been previously linked to prostate cancer metastasis by other groups. In human prostate cancer tissues, we showed that SAFB1 protein levels decline with progression to metastasis, consistent with an AR suppressive function. Stable silencing of SAFB1 in LNCaP cells resulted in upregulation of AR and the AR-regulated gene, prostate specific antigen (PSA). SAFB1-silenced LNCaP cells also showed a dramatic suppression of androgen sensitivity. In vivo experiments on the effect of stable SAFB1 silencing are now in progress. However, our current data set already strongly suggests that SAFB1 is capable of regulating androgen-regulated gene expression ***on a genome-wide scale***. Our findings suggest that loss of expression of the SAFB1 protein, which we have shown occurs with progression to castrate-resistant prostate cancer (CRPC) in humans, alters the ability of AR to function as a transcriptional regulator. Our data provide evidence that SAFB1 serves to integrate signaling from the Mst1 and Akt1 pathways with the AR transcriptional machinery.

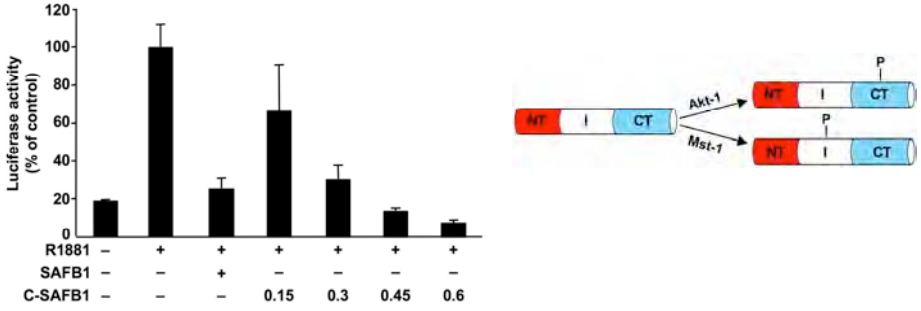


Figure 1. Enforced expression of SAFB1 in LNCaP cells inhibits androgen-responsive gene expression. The c-terminal domain of SAFB1, which contains an Mst1 binding site, is sufficient for AR inhibition to occur. Biochemical data (not shown) have identified the domains on SAFB1 phosphorylated by Akt1 and Mst1 kinases (cartoon).

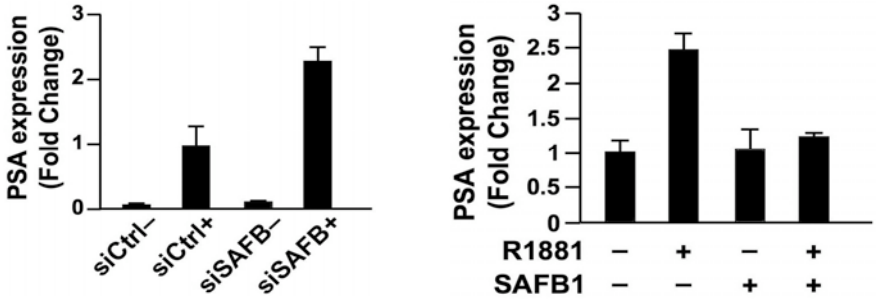
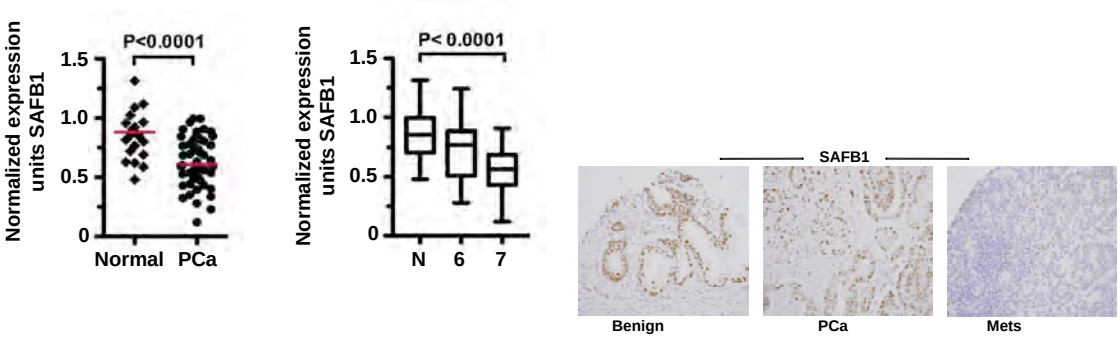


Figure 2. SAFB1 silencing increases PSA expression, while enforced SAFB1 inhibits PSA expression.



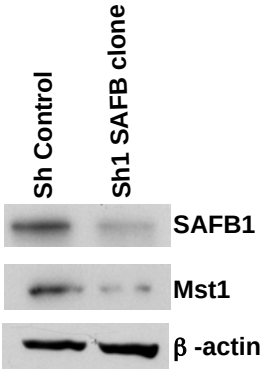
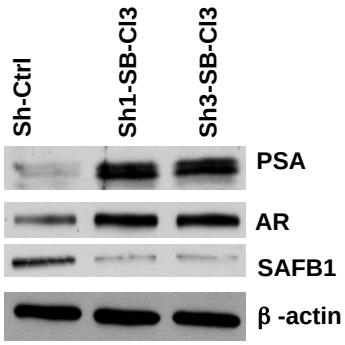


Figure 3. SAFB1 expression declines with prostate cancer progression. SAFB1 protein levels are positively correlated with Mst1 levels. Silencing of SAFB1 lowers results in reduced expression of Mst1.

A



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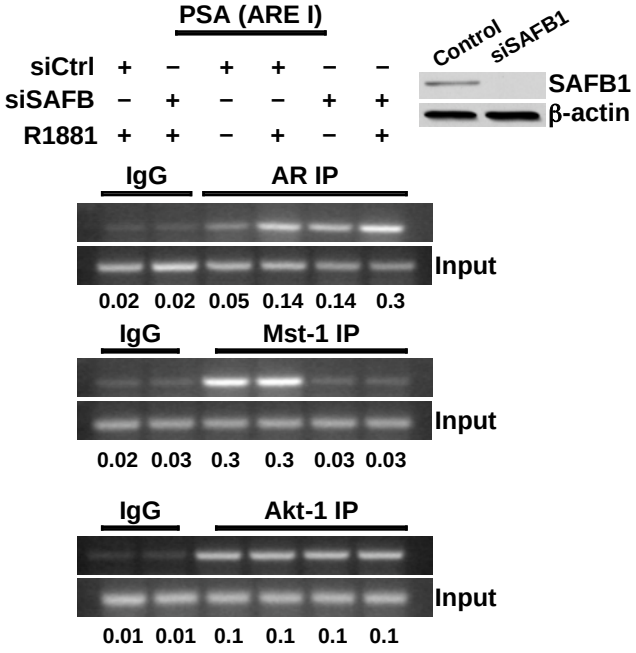

Figure 4. **A.** Stable silencing of SAFB1 results in upregulation of PSA and androgen receptor protein expression. **B.** Silencing of SAFB1 inhibits Mst1 but not Akt1 association with chromatin at the PSA promoter.

REPORTABLE OUTCOMES

Mukhopadhyay, M., Kim, J., Cinar, B., Ramachandran, A., Hager, M.H., Di Vizio, D., Adam, R.M., Rubin, M.A., Raychaudhuri, P., De Benedetti, A., and Freeman, M.R**.** (2009) Heterogeneous nuclear ribonucleoprotein K is a novel regulator of androgen receptor translation. Cancer Res 69:2210-2218.

Di Vizio, D., Kim, J., Hager, M.H., Morello, M., Yang, W., Lafargue, C.J., True, L., Rubin, M.A., Adam, R.M., Beroukhim, R., Demichelis, F., and Freeman, M.R. (2009) Oncosome formation in prostate cancer: Association with a region of frequent chromosomal deletion in metastatic disease. Cancer Res 69:5601-5609.

Freeman, M.R., Di Vizio, D., Solomon, K.R. (2010) The Rafts of the Medusa: cholesterol targeting in cancer therapy. Oncogene 29:3745-3747.

CONCLUSIONS AND SIGNIFICANCE

This study has identified the RNA binding protein hnRNP-K as a physiologically relevant regulator of the androgen receptor (AR) in prostate cancer cells. As a result of funding from the DoD, we have now identified an hnRNP-K interacting protein, the nuclear matrix protein SAFB1, as a novel AR regulator that functions at the level of chromatin. In year 2 of this project we have shown that SAFB1 is a substrate for the Akt1 and Mst1 kinases and therefore is a potential signaling node where apoptotic and pro-survival signals converge with the androgenic transcriptional apparatus. We have demonstrated that SAFB1 levels decline with disease progression in human prostate cancer. Current evidence suggests that this alteration is likely to result in widespread changes across the genome in the AR-regulated transcriptome. These results *suggest that SAFB1 is a novel* ***inhibitor*** *of the progression to castration-resistant prostate cancer (CRPC).* We are currently exploring the biological and genomic consequences of this exciting and highly novel finding, including the relationship to membrane cholesterol and lipid metabolism, and the relationship between the SAFB1/Akt1/Mst1 complex in the nucleus and plasma membrane Mst1/Akt1 complexes we have previously described (Cinar et al. 2007).

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Personnel Receiving Salary from W81XWH-08-1-0150

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Oncogene (2010) 29, 3745–3747
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COMMENTARY

The Rafts of the Medusa: cholesterol targeting in cancer therapy

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In this issue of *Oncogene*, Mollinedo and co-workers present promising evidence that cholesterol-sensitive signaling pathways involving lipid rafts can be therapeutically targeted in multiple myeloma. Because the pathways considered in their study are used by other types of tumor cells, one implication of this report is that cholesterol-targeting approaches may be applicable to other malignancies.
Oncogene (2010) **29,** 3745–3747; doi:10.1038/onc.2010.132; published online 3 May 2010

Cholesterol is a sterol that serves as a metabolic precursor to other bioactive sterols, such as nuclear receptor ligands, and also has a major role in plasma membrane structure. Cholesterol and long-chain sphingolipids are believed to accumulate in 'liquid-ordered' patches termed lipid rafts, which are distinct in dynamic behavior from cholesterol-poor regions of the membrane. Biophysical, biochemical and imaging studies have provided strong evidence that lipid rafts exist in biological membranes, although details of their precise form and stability are still hotly debated. Cholesterol-rich microdomains have been shown to sequester a variety of membrane-associated signaling proteins, while excluding others, and a variety of approaches have shown that cholesterol itself has a role in transmitting cell growth, survival and differentiation signals. There has been speculation that cholesterol-dependent membrane dynamics, such as receptor clustering, may be targeted therapeutically in the case of certain malignancies.

Published evidence suggests that a cholesterol-focused approach might work in some clinical scenarios. Cholesterol-lowering drugs that inhibit the enzyme HMG-CoA reductase, which catalyzes the rate-limiting step in cholesterol synthesis (these drugs are generically termed 'statins'), have been reported to inhibit cancer incidence or progression in some studies. Although there is much controversy, buttressed by claims and counterclaims, in the various population-based reports of the effects of statins on cancer, recent evidence published by several groups examining large prospective series suggest that prostate cancer progression is likely to be inhibited by long-term cholesterol-lowering therapy (Platz *et al*., 2006; Mondul *et al*., 2010). These promising results in humans are in agreement with animal model data, in which cholesterol is raised or lowered and prostate tumor growth thereby promoted or inhibited, respectively (Zhuang *et al*., 2005; Solomon *et al*., 2009). Prostate cancer may be a special case, however, because recent observations from several groups indicate that tumor cells are capable of *de novo* androgen synthesis from cholesterol, obviating the need to acquire the hormone from the circulation. Because androgens are generally thought to promote prostate cancer disease progression, the relative clarity of the epidemiological data in prostate cancer in comparison to other organ sites may arise from the role of cholesterol as the synthetic precursor of androgens.

The paper by Mollinedo *et al*. in this issue, along with previous studies from this group and from other labs, provide persuasive evidence that membrane cholesterol itself is a critical mediator of cell survival signaling mechanisms that can be effectively targeted with clinically relevant drugs. Because this has been shown in multiple myeloma, a malignancy without the hormone dependence of prostate cancer, the *in situ* production of steroid hormones is an unlikely explanation for the sensitivity to cholesterol-targeting manipulations. Edelfosine, and the related compound, perifosine, are prototypic members of a family of synthetic lipids with tumor-killing properties known as alkyl-lysophospholipid analogs. Both drugs are presently in human trials and significant clinical responses have been reported. In a series of studies, edelfosine was shown to accumulate in cell membrane rafts and cause co-clustering of components of the death-inducing signaling complex, resulting in tumor cell apoptosis.

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Received 14 March 2010; accepted 14 March 2010; published online 3 May 2010

Raft disruption by cholesterol depletion, using a variety of distinct approaches, inhibits drug uptake by tumor cells, preventing apoptosis. This inhibition is reverted by cholesterol replenishment. *in vivo*, edelfosine accumulates in mouse plasmacytomas, inducing apoptosis. However, the drug uptake by the tumor is reduced if tumor cholesterol levels are lowered by statin therapy, resulting in treatment failure. These and supporting observations strongly suggest that tumor apoptosis induced by the alkyl-lysophospholipid compounds is cholesterol dependent.

Edelfosine was the first compound reported to promote apoptosis by ligand-independent activation of Fas/CD95 death receptors. However, this class of drug may also operate by cholesterol-sensitive mechanisms distinct from death-inducing signaling complex formation. Perifosine was shown to potently inhibit activation of the serine-threonine kinase Akt (Hideshima *et al*., 2006), which usually performs a pro-survival function. In other studies, Akt has been identified as a cholesterol-sensitive protein that localizes to lipid rafts (Adam *et al*., 2007), consistent with a mechanism of action where perifosine alters raft-mediated signaling from Akt. Collectively, these results suggest that cholesterol/lipid raft targeting may cooperatively activate both pro-death and inhibit pro-survival pathways simultaneously.

One important issue is that edelfosine is taken up specifically by $CD138^+$ malignant cells obtained from patients with multiple myeloma, whereas $CD138^-$ normal cells from the same patients and normal peripheral blood lymphocytes did not incorporate the drug. Similarly, edelfosine induced clustering of Fas/CD95 and TRAIL receptors in lipid rafts in tumor cells, but not in normal cells from the same patients. *In vivo*, edelfosine concentrated in the multiple myeloma tumors but the drug localized poorly in liver and kidney. These observations suggest that tumor cell membranes are substantially altered in lipid composition, a consequence of malignant transformation that could be exploited with lipid-targeting agents.

Lipid targeting in cancer therapy is of growing interest because of a reawakened appreciation for the role of metabolic pathways as essential features of malignant transformation and progression (Vander Heiden *et al*., 2009). Tumor cells consume high levels of glucose but often simultaneously suppress ATP production, diverting carbon atoms and reducing equivalents toward pathways that synthesize macromolecules, particularly lipids required for new membrane formation. Upregulation of fatty acid synthase, the major source of long-chain fatty acids (principally palmitate) in tumor cells, is a prominent characteristic of this metabolic profile (Menendez and Lupu, 2007). Lipogenesis is also activated by Akt upregulation, another common event in malignancy. Preclinical data have shown that fatty acid synthase is effectively targetable *in vivo* and efforts are underway to improve the toxicity profile associated with the available anti-fatty acid synthase compounds. Inhibiting various components of the growth factor receptor$\rightarrow$PI3-kinase$\rightarrow$Akt$\rightarrow$mTOR pathway, an active area in pharmaceutical R&D, will produce major alterations in lipid metabolism, only some of which are known. The landscape of tumor cell membranes is drastically altered from normal as a result of shifts in metabolism arising from malignant transformation; however, characterization of membrane lipid composition in tumor cells is still at a fairly primitive stage. Given the absolute requirement for cholesterol in the synthesis of mammalian cell membranes, it follows that rapidly proliferating tumor cells require more cholesterol than normal cells. Moreover, the ability of cancer cells to metastasize may depend on the formation of cholesterol-rich cell extensions called invadopodia, which may not form in the absence of excess cholesterol (Caldieri and Buccione, 2010). Thus, metastatic cells' dependence on abnormal levels of cholesterol may prove to be their undoing if vulnerabilities in lipid metabolism can be identified and exploited. As the tumor metabolism picture becomes clearer over the next decade, lipid-targeting strategies should prove relevant to the clinical situation, at least for some classes of tumors. Because of shared metabolic consequences of cell transformation that arise from the many varieties of genetic alterations in multiple signal transduction pathways, one can even imagine that lipid-targeting approaches may ultimately prove to be more widely efficacious than is currently the case for drugs directed toward the major protein signal transduction targets, such as ErbB family receptors and other kinases.

There are some caveats that should be considered when evaluating the fortunes of cholesterol targeting. Modeling cholesterol reduction in rodents is challenging because statin drugs do not lower circulating cholesterol in mice and rats, as they do in humans. Cholesterol lowering in rodent tumors will thus depend on whether the drug can penetrate sufficiently into the tumor bed, a scenario that does not adequately model the conventional cholesterol reduction situation in humans, in which the level of extrahepatic statin is low and of short duration. However, other approaches to cholesterol lowering in mice have recently been developed (Solomon *et al*., 2009) and these might be exploited to identify cholesterol-sensitive tumor types by way of xenografts or transgenic models. Whether the unusually high level of selectivity toward tumor cells seen with edelfosine will be replicated with other lipid-targeting agents is an open question. In addition, high concentrations of cholesterol are naturally found in the brain, ilium, liver and prostate. Consequently, a cholesterol-targeting approach may not be selective for tumors at certain anatomical sites. It is also not clear whether edelfosine can cross either the blood–brain or blood–testes barriers, so malignancies at these locations may not be targetable by the alkyl-lysophospholipid class of compounds.

A not unfitting metaphor for cancer, Medusa was a mythical

creature, highly feared, with snakes in place of hair, which could turn someone to stone with a single glance. The Raft of the Medusa (Le Radeau de la Méduse) is a famous oil painting by Théodore Géricault, which depicts a tragic escape from a wrecked French naval vessel of the same name. Although most of the castaways lost their lives in the escape attempt, some were saved by the hastily constructed raft. It is intriguing that the metaphor of the Raft of the Medusa providing sanctuary and life may someday extend to patients, where the cholesterol- and lipid-rich membrane environment of tumors might provide effective therapeutic opportunities.

Conflict of interest

The authors declare no conflict of interest.

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