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REVIEW

A mitochondrial based oncology platform for targeting cancer stem cells (CSCs): MITO-ONC-RX

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ABSTRACT

Here, we wish to propose a new systematic approach to cancer therapy, based on the targeting of mitochondrial metabolism, especially in cancer stem cells (CSCs). In the future, we envision that anti-mitochondrial therapy would ultimately be practiced as an add-on to more conventional therapy, largely for the prevention of tumor recurrence and cancer metastasis. This mitochondrial based oncology platform would require a panel of FDA-approved therapeutics (e.g. Doxycycline) that can safely be used to inhibit mitochondrial OXPHOS and/or biogenesis in CSCs. In addition, new therapeutics that target mitochondria could also be developed, to optimize their ability to eradicate CSCs. Finally, in this context, mitochondrial-based biomarkers (i.e. “Mito-signatures”) could be utilized as companion diagnostics, to identify high-risk cancer patients at diagnosis, facilitating the early detection of tumor recurrence and the prevention of treatment failure. In summary, we suggest that new clinical trials are warranted to test and possibly implement this emerging treatment strategy, in a variety of human cancer types. This general approach, using FDA-approved antibiotics to target mitochondria, was effective in killing CSCs originating from many different cancer types, including DCIS, breast (ER(+) and ER(-)), prostate, ovarian, lung and pancreatic cancers, as well as melanoma and glioblastoma, among others. Thus, we propose the term MITO-ONC-RX, to describe this anti-mitochondrial platform for targeting CSCs. The use of re-purposed FDA-approved drugs will undoubtedly help to accelerate the clinical evaluation of this approach, as these drugs can move directly into Phase II clinical trials, saving considerable amounts of time (10–15 y) and billions in financial resources.

1. Mitochondrial function is required for CSC propagation

Tumors and their microenvironment are incredibly heterogeneous structures, which behave like metabolic ecosystems [1–12]. In accordance with this cellular and metabolic heterogeneity, it is now well accepted that more than a single type of cancer cell exists [1–12]. For example, within a given epithelial cancer cell line (such as MCF7), there are “bulk” cancer cells (~ 85–95%; the majority of the population), as well as various types of progenitor cells (< 5%), and cancer stem cells (CSCs; < 1%). CSCs and progenitor cells are thought to be the most dangerous, as they behave as tumor-initiating cells (TICs) in vivo and can undergo metastasis; in contrast, “bulk” cancer cells are largely non-tumorigenic.

Because CSCs are so “rare”, very little is actually known about their metabolic properties. To functionally enrich for CSCs, the entire cell population can be trypsinized and seeded as a single-cell suspension onto low-attachment plates [1,2]. Under these conditions, the majority of “bulk” cancer cells die (> 90%) via apoptosis, while only the CSCs survive and propagate, ultimately resulting after 5 d in the formation of 3D spheroid structures. Under these conditions, each 3D spheroid is clonally formed from a single CSC. For breast CSCs, these 3D spheroids are also known as tumor-spheres or mammo-spheres [1–12]. The generation of these 3D spheroids is thought to mimic the process of tumor formation and/or metastasis, providing an excellent model for drug discovery and functional validation.

Thus, to begin to understand the potential metabolic differences between “bulk” cancer cells...
and CSCs, we first compared cultured breast cancer cells grown either as monolayers or 3D spheroids. Then, they were subjected to profiling, via unbiased label-free proteomics analysis [13].

As a result of this molecular comparison, it became clear that over 60 nuclear-encoded mitochondrial proteins were specifically up-regulated in 3D spheroid structures, relative to monolayer cells processed in parallel [13]. Virtually identical results were obtained with two distinct ER(+) breast cancer cell lines (MCF7 and T47D; > 40 overlapping mitochondrial proteins). Informatics analysis of the list of up-regulated mitochondrial proteins was consistent with an increase in mitochondrial mass, due either to i) increased mitochondrial biogenesis or ii) a shut down in mitophagy, or both. As such, these results indicated that high mitochondrial mass is a new characteristic feature of the CSC phenotype [13]. These results also suggested the testable hypothesis that CSCs are critically dependent on OXPHOS and/or new mitochondrial biogenesis (protein translation), for their survival and propagation. In accordance with this hypothesis, 3D spheroid formation was effectively blocked using specific mitochondrial inhibitors, such as oligomycin, which targets mitochondrial Complex V and shuts off ATP synthesis [13]. However, oligomycin is quite toxic, so it cannot be used as an anti-cancer therapeutic.

As such, the question remains: How can we safely target mitochondria in CSCs, without inducing serious side effects in normal cells?

MitoTracker is a non-toxic fluorescent probe that can be used to directly measure mitochondrial mass in live cells by flow cytometry [14]. In order to further validate the functional relationship between high mitochondrial mass and “stemness”, we employed staining with MitoTracker to metabolically fractionate the MCF7 cell line into “Mito-high” and “Mito-low” cell sub-populations [14]. For this purpose, we used MitoTracker Deep Red FM, which is a far-red fluorescent dye (abs/em ~ 644/665 nm) that stains mitochondria in live cells. As predicted the “Mito-high” cell population, with increased mitochondrial mass, showed the greatest capacity for i) 3D spheroid formation and ii) tumor initiation in a pre-clinical animal model in vivo [14]. Therefore, mitochondrial mass is a critical determinant of stemness in cancer cells. In addition, elevated telomerase activity (hTERT), a functional marker of proliferation and immortality in CSCs, was also specifically associated with high mitochondrial mass [15].

In light of the above results, a targeted reduction in mitochondrial mass or OXPHOS should provide a specific Achilles’ heel for the eradication of CSCs [13–15].

2. Mitochondrial-based therapeutic strategies for eradicating CSCs: FDA-approved drug repurposing and natural products

Over the last several years, our laboratory has focused on the identification and repurposing of FDA-approved drugs that can be used to inhibit the propagation of CSCs. These different antibiotics include members of the i) Tetracycline family (Doxycycline/Tigecycline) [16] and the ii) Erythromycin family (Azithromycin) [16], as well as iii) anti-parasitic drugs (Pyrvinium pamoate and Atovaquone) [16,17] and iv) anti-microbials targeting drug-resistant mycobacterium (Bedaquiline; TB, tuberculosis) [18], (Figure 1, and Table 1).

One aspect that all these antibiotics have in common is that they exert manageable anti-mitochondrial side-effects, which can be repurposed as their therapeutic effects, to eradicate CSCs [19,20]. More specifically, Doxycycline and Azithromycin inhibit mitochondrial protein translation, effectively blocking new mitochondrial biogenesis [16–20]. Since most tissues already have large numbers of mitochondria (especially skeletal muscle, heart and brain), normal cells remain unaffected. In contrast, CSCs appear to be “addicted” to the generation of new mitochondria, providing a novel basis for the selectivity of these antibiotics in targeting CSCs. New mitochondrial biogenesis may also be required for asymmetric cell division in CSCs.

In addition, Pyrvinium pamoate and Atovaquone behave as mitochondrial OXPHOS inhibitors, affecting Complexes II and III [16,17]. Bedaquiline inhibits the mitochondrial ATP-synthase (Complex V) [18]. Finally, we also identified a series of experimental and natural compounds targeting CSCs, including glycolysis inhibitors (Vitamin C and Silibinin) [20] and mitochondrial inhibitors (Actinonin; CAPE, from
Honey bee propolis) [20], as well as inhibitors of protein synthesis (puromycin) and NAD(+) recycling (FK-866) [20,21] (Figure 2 and 3).

As CSCs appear to be highly proliferative, due to their over-expression of telomerase (hTERT), they are quite sensitive to Palbociclib, an FDA-approved CDK4/6 inhibitor, with an IC-50 of ~ 100 nM [15] (Table 1). Therefore, inhibition of CSC proliferation is an alternative or could be used in conjunction with anti-mitochondrial therapy [15].

Regarding therapy with Doxycycline, one possible concern that arises is the potential for the development of drug-resistance in CSCs [22]. To address this issue directly, we developed and characterized the phenotypic behavior of Doxy-resistant (DoxyR)-MCF7 cells [22]. Remarkably, what we observed is that DoxyR-CSCs show a dramatic shift towards aerobic glycolysis, due to a loss of mitochondrial function, ultimately resulting in metabolic inflexibility [22]. As expected, they showed an up to 35-fold loss of mitochondrial-DNA encoded proteins (mt-DNA) that are absolutely required for OXPHOS activity, such as MT-ND3, MT-CO2, MT-ATP6 and MT-ATP8 [22]. Also, DoxyR-CSCs appeared to be more “quiescent”, with > 50% reduc-

### Table 1. FDA approved drugs successfully used to eradicate CSCs.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Inhibition of</th>
<th>FDA-approved</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>Mito Biogenesis</td>
<td>Yes</td>
<td>[16,19,22]</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>Mito Biogenesis</td>
<td>Yes</td>
<td>[16]</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Mito Biogenesis</td>
<td>Yes</td>
<td>[16]</td>
</tr>
<tr>
<td>Pyrvinium</td>
<td>OXPHOS/Complex II</td>
<td>Yes</td>
<td>[16]</td>
</tr>
<tr>
<td>Pamoate</td>
<td>OXPHOS/Complex III</td>
<td>Yes</td>
<td>[17]</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>Complex V</td>
<td>Yes</td>
<td>[18]</td>
</tr>
<tr>
<td>Palbociclib</td>
<td>CDK4/6</td>
<td>Yes</td>
<td>[15]</td>
</tr>
</tbody>
</table>

**Figure 1.** Repurposing FDA-approved antibiotics for targeting mitochondria in CSCs. a) Doxycycline; b) Azithromycin; c) Pyrvinium (pamoate salt; not shown); d) Atovaquone; and e) Bedaquiline. Doxycycline and Azithromycin (a,b) are known to inhibit mitochondrial protein translation as an off-target side effect. They are used clinically as antibiotics to inhibit bacterial protein synthesis. Similarly, Pyrvinium pamoate and Atovaquone (c,d) are known to inhibit OXPHOS (related to mitochondrial complex II/III), as a side effect. Bedaquiline (e) was originally designed to inhibit the bacterial ATP-synthase, which is analogous to mitochondrial complex V. All of these FDA-approved drugs (a-e) have been shown to inhibit the anchorage-independent propagation of CSCs, by targeting mitochondrial function.

**Figure 2.** Naturally Occurring Compounds

a) Caffeic acid phenyl ester (CAPE); b) Vitamin C (Ascorbic acid); c) Actinonin and d) Silibinin. CAPE (from bee propolis) inhibits OXPHOS; Vitamin C blocks GAPDH (a glycolytic enzyme), which is directly upstream of mitochondrial metabolism; Actinonin inhibits the initiation of both bacterial and mitochondrial protein translation; Silibinin (from milk thistle) decreases glucose uptake, by targeting the GLUT family of transporters. These natural products all inhibit CSC expansion.
tions in proliferation and cell migration, as well as a significantly impaired ability to form 3D spheroids. As a consequence, our results showed that DoxyR-CSCs are especially sensitive to other metabolic therapies, including inhibitors of i) OXPHOS (Atovaquone, Irinotecan, Sorafenib, Niclosamide), ii) glycolysis (Vitamin C and Stiripentol) and iii) autophagy (Chloroquine).

Importantly, many of these drugs are already clinically-approved (Atovaquone, Irinotecan, Sorafenib, Niclosamide, Stiripentol, Chloroquine) or they are natural products (Vitamin C and Berberine), making them readily available for clinical trials (Table 2). Therefore, the efficacy of Doxycycline could be potentially improved, by developing combination therapies with these other metabolic inhibitors, based on the concepts of metabolic inflexibility and synthetic lethality in cancer cells [22].

### 3. Mitochondrial-based therapeutic strategies for eradicating CSCs: developing new mitochondrial inhibitors

Because of the success of our drug repurposing efforts, we also decided to develop a series of new therapeutics, by focusing on key mitochondrial targets [23–26]. These results are briefly summarized in Table 3.

As a consequence, based on in silico drug design (i.e. computational chemistry) and phenotypic drug screening, we identified several new families of mitochondrial inhibitors, termed Mitoriboscins [23], (Figure 4). They were specifically designed to target the mitochondrial ribosome, to inhibit mitochondrial protein synthesis. By targeting the mitochondrial enzymes OXCT1 and ACAT1, we also developed new mitochondrial inhibitors that interfere with ketone metabolism, by mimicking the structure of CoA [24], (Figures 5 and 6). In addition, we identified a novel approach to acutely induce a Vitamin B2 (riboflavin) deficiency, that potently inhibits CSC propagation, with an IC-50 of ~ 3 nM [25], (Figure 7, Upper). Thus, this drug is approximately 30 times more potent than Palbociclib for targeting CSCs.

### Table 2. Nine drugs, or nutraceuticals, that were successfully used in conjunction with Doxycycline, to eradicate CSCs.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Target</th>
<th>FDA-approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atovaquone</td>
<td>OXPHOS</td>
<td>Yes</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>OXPHOS</td>
<td>Yes</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>OXPHOS</td>
<td>Yes</td>
</tr>
<tr>
<td>Niclosamide</td>
<td>OXPHOS</td>
<td>Yes</td>
</tr>
<tr>
<td>Berberine</td>
<td>OXPHOS</td>
<td>Natural supplement</td>
</tr>
<tr>
<td>2-DG*</td>
<td>Glycolysis</td>
<td>Experimental</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Glycolysis</td>
<td>Natural supplement</td>
</tr>
<tr>
<td>Stiripentol</td>
<td>Glycolysis</td>
<td>Clinically-approved (Europe/Canada/Japan)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Autophagy</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*2-deoxy-glucose; See reference [22], for additional information.

### Table 3. Novel anti-mitochondrial drug design: Four new therapeutic classes.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Target</th>
<th>Metabolic Process</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitoriboscins</td>
<td>Mito-ribosome</td>
<td>Mito Protein Synthesis</td>
<td>[23]</td>
</tr>
<tr>
<td>Mitoketoscins</td>
<td>OXCT1/ACAT1</td>
<td>Ketone Metabolism</td>
<td>[24]</td>
</tr>
<tr>
<td>Mitoflavoscins</td>
<td>Mito Complex I/II</td>
<td>Flavin-containing proteins</td>
<td>[25]</td>
</tr>
<tr>
<td>TPP*</td>
<td>Mitochondria</td>
<td>Mito-targeting-signal(s)</td>
<td>[26]</td>
</tr>
</tbody>
</table>

*Tri-phenyl-phosphonium
The use of tri-phenyl-phosphonium (TPP) also appears to be a very promising approach, as it behaves as a chemical mitochondrial targeting signal (MTS) (Figure 7, Lower) \[26\]. Interestingly, a series of TPP compounds we tested were non-toxic in normal fibroblasts, but were selectively toxic in “bulk” cancer cells and CSCs \[26\]. Therefore, these TPP compounds appear to be able to metabolically distinguish between “normal cell” mitochondria and “malignant” mitochondria \[26\]. Since “bulk” cancer cells and CSCs likely have mitochondria with higher membrane potentials, this could explain the selective targeting of cancer cell mitochondria with TPP compounds.

Finally, we have begun to search for new naturally-occurring mitochondrial inhibitors, that can be used to more effectively target CSCs (Table 4). In this context, we recently showed that two molecules that are novel components of Bergamot, namely Brutieridin and Melitidin, act as specific Statin-like drugs and inhibit mevalonate metabolism, as well as CSC propagation (Figure 8) \[27\]. Interestingly, Bergamot is currently used as a fragrance in many health and well-being products (soaps and perfumes), and is a significant component of Earl Grey English breakfast tea.

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Interference with the normal process of mitochondrial fission-fusion cycles, by targeting the DRP1 protein, may also represent a new viable strategy for eradicating CSCs (See Figure 8 and Table 4) [28].

4. Mitochondrial inhibitors also show broad-spectrum antibiotic activity

The identification and design of new mitochondrial inhibitors has other interesting medical applications and benefits, such as the development of new anti-bacterial and anti-fungal agents, to combat antibiotic-resistance.

According to the “Endo-symbiotic Theory of Mitochondrial Evolution”, mitochondria first originated historically from the engulfment of aerobic bacteria, an event that occurred ~ 1.45 billion years ago [29–32]. As a result, mitochondria still share strong structural and functional similarities with bacteria, explaining the off-target effects of antibiotics, which often show manageable mitochondrial side-effects [29–32]. Conversely, it would then be predicted that

**Table 4.** Other experimental mitochondrial inhibitors for targeting CSCs.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Target</th>
<th>Metabolic Process Inhibited</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDIVI1</td>
<td>DRP1*</td>
<td>Mitochondrial Fission/Fusion</td>
<td>[28]</td>
</tr>
<tr>
<td>Brutieridin</td>
<td>HMGR**</td>
<td>Mevalonate Metabolism; Statin-like</td>
<td>[27]</td>
</tr>
<tr>
<td>Melitidin</td>
<td>HMGR**</td>
<td>Mevalonate Metabolism; Statin-like</td>
<td>[27]</td>
</tr>
</tbody>
</table>

*a*Dynamin-related protein 1  
**3-hydroxy-3-methylglutaryl-CoA-reductase.
mitochondrial inhibitors may also show some moderate anti-bacterial and anti-fungal side effects [29–32].

To directly test this hypothesis, we evaluated the anti-bacterial and anti-fungal activity of the Mitoriboscins [23]. Interestingly, several Mitoriboscins showed anti-bacterial activity towards both gram-positive and gram-negative organism(s), as well as pathogenic yeast (Candida albicans) and even Methicillin-resistant Staphylococcus aureus (MRSA) [23]. Therefore, this systematic approach, using cancer cells for initial drug screening, may also be useful for developing new antibiotics, to combat drug-resistant micro-organisms [23].

5. Mitochondrial-based companion diagnostics: treatment stratification and predicting the response to therapy

Over one thousand mitochondrial proteins are encoded by the nuclear genome. Therefore, we have begun to assess their potential prognostic value as novel biomarkers and companion diagnostics. Our hypothesis was that the over-expression of a given mitochondrial protein in cancer cells and CSCs may be associated with tumor recurrence and metastasis, due to the emergence of drug resistance, ultimately resulting in treatment failure (Figure 9).

To test this hypothesis, we used an online survival-analysis tool (known as the KM-plotter; http://kmplot.com/analysis/) to perform Kaplan-Meier (K-M) studies on > 400 nuclear mitochondrial gene transcripts, to interrogate publically available microarray data from patients with four distinct epithelial cancer types: i) breast, ii) ovarian, iii) lung and iv) gastric [32,33]. This approach allowed us to directly perform in silico validation of these potential mitochondrial biomarkers [33,34].

Remarkably, in all four anatomic cancer types, we observed that the over-expression of mitochondrial gene transcripts was specifically associated with poor clinical outcome. Importantly, this

![Figure 9](image-url)

Figure 9. Identifying mitochondrial-based companion diagnostics for more personalized cancer therapy. Briefly, mitochondrial markers could be used to stratify cancer patients into high-risk and low-risk groups, at diagnosis. Patients with high levels of mitochondrial markers (“bad prognosis”) could be treated with mitochondrial-based therapies, as an add-on to the standard of care, in order to effectively prevent tumor recurrence, metastasis and drug-resistance. Modified from [32,33].

![Figure 10](image-url)

Figure 10. A short mitochondrial gene signature predicts tumor recurrence and distant metastasis, in high-risk breast cancer patients, receiving endocrine therapy. Note that high expression of this Mito-Signature predicts treatment failure on Tamoxifen (Tam). These represent ER-positive patients, with the luminal A sub-type of breast cancer, who showed local lymph node metastasis at diagnosis, with 10–15 y of follow-up data. RFS, recurrence-free survival; DMFS, distant metastasis-free survival. Reproduced with permission from [32].
approach effectively predicted tamoxifen-resistance in ER(+) breast cancer patients (Figure 10), as well as Taxol- and Platin-resistance in ovarian cancer patients (Figure 11) [32,33]. These results are functionally supported by further experimental observations demonstrating that Tamoxifen-resistant MCF7 cells (TAMR) show a significant increase in mitochondrial oxygen consumption and ATP production [35].

Therefore, in the future, we propose that high-risk patients could be first identified at diagnosis, by the high expression of mitochondrial markers or “Mito-signatures” in their primary tumors [33,34]. Here, we define “Mito-signatures” as combinations of mitochondrial markers (mRNA levels) that can be used to more accurately predict clinical outcome in patient populations.

Then, these patients could be also treated with existing FDA-approved antibiotics or novel mitochondrial therapeutics, as an add-on to conventional anti-cancer therapy [33,34].

6. Other advantages of anti-mitochondrial therapy with Doxycycline

Doxycycline shows many other interesting anti-cancer properties that should be further explored. For example, Doxycycline behaves as a radio-sensitizer, making CSCs approximately 3 to 5 times more sensitive to radiation treatment [36]. In addition, Doxycycline effectively targets hypoxic CSCs and overcomes Paclitaxel-resistance, under conditions of hypoxia; this may have important implications for achieving more effective anti-angiogenic therapy [37]. Finally, Doxycycline appears to be effective as a mutation-independent approach for targeting CSCs, as it inhibits both activated H-Ras (G12V) and c-Myc oncogenes, as well as other environmental oncogenic stimuli (mitochondrial oxidative stress/ROS), via the specific targeting of mitochondrial biogenesis [38].

7. Conclusions

In conclusion, we propose a new, integrated, multidisciplinary, medical oncology platform to target cancer stem cells (CSCs), in most cancer types. This new mitochondrial oncology platform, known as MITO-ONC-RX, consists of both therapeutic and diagnostic modalities (Figure 12). As increased mitochondrial biogenesis and OXPHOS are key characteristics of CSCs, we propose to target metabolism in CSCs, using FDA drug repurposing and/or new first-in-class therapeutics, such as the i) Mitoriboscins, the ii) Mitoketoscins and the iii) Mitoflavoscins, as well as iv) TPP-based compounds. In this context, we have also discovered a series of mitochondrial markers (companion diagnostics) for identifying and selecting.
the high-risk cancer patient population that is most likely to benefit from anti-mitochondrial therapy.

The essential role of mitochondria in the propagation of CSCs has now been confirmed and extended by other laboratories, in London and at MD Anderson Cancer Center, as well as other institutions (See references #2 and #39, for specific citations). In further support of this notion, please see the following articles for further reading on the subject [39–45].

Acknowledgments

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Author contributions

Professors Lisanti and Sotgia conceived and prepared the initial draft of this review article, including the figures and tables. This draft manuscript was then further edited, by Drs. Bela Ozsvari, Marco Fiorillo, Ernestina Marianna De Francesco and Gloria Bonuccelli.

Disclosure statement

MPL and FS hold a minority interest in Lunella Biotech, Inc.

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Mitochondrial fission as a driver of stemness in tumor cells: mDIVI1 inhibits mitochondrial function, cell migration and cancer stem cell (CSC) signalling. Oncotarget. 2018 Jan 19;9(17):13254–13275.


