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Therapeutic potential of mTOR inhibitors for targeting cancer stem cells

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The mammalian target of rapamycin (mTOR) pathway is aberrantly activated in many cancer types. As the intricate network of regulatory mechanisms controlling mTOR activity is uncovered, more refined drugs are designed and tested in clinical trials. While first generation mTOR inhibitors have failed to show clinical efficacy due partly to the feedback relief of oncogenetic circuits, newly developed inhibitors show greater promise as anti-cancer agents. An effective drug must defeat the cancer stem cells (CSCs) while sparing the normal stem cells. Due to its opposing role on normal and malignant stem cells, mTOR lends itself very well as a therapeutic target. Indeed, a preferential inhibitory effect on CSCs has already been shown for some mTOR inhibitors. These results provide a compelling rationale for the clinical development of mTOR-targeted therapies.

Introduction

Cancer remains the second most common cause of death in the US. Despite notable improvements in survival over the past three decades for most cancer types, an estimated 589 430 cancer deaths will still occur in the United States this year [1]. The high cancer death rate is often due to diagnosis during late stages of disease and a lack of specific treatments for advanced stages of cancer. Nevertheless, although traditional, non-specific cytostatic chemotherapy remains the treatment of choice for many malignancies, as the genetics of cancer are unraveed [2], more effective molecularly targeted drugs are under development. First tested in animal models of cancer, and then in humans, these drugs are creating enthusiasm and hope that cancer will be defeated in the near future.

Among the molecular targets for cancer therapy is the mammalian target of rapamycin (mTOR) pathway. mTOR is aberrantly activated in many cancer types, including glioblastoma [3] and cancers of the breast [4], pancreas [5], colon [6], prostate [7] and ovary [8]. Although preclinical studies suggested that mTOR inhibition could provide synergistic benefits when added to other targeted signal transduction inhibitors [9], subsequent studies have failed to demonstrate clinical efficacy [10].

In this review, we will first discuss different models to explain cancer origin, maintenance and evolution. We will then discuss the development of mTOR inhibitors as a novel class of anticancer agents, their activity against cancer cells bearing stem cell-like features and some of the major challenges of personalized-medicine.

Targeted therapies, tumour evolution and drug resistance: implications for therapy

In the last decade, impressive steps towards understanding the biology of cancer have been accomplished, thanks to the advances of next generation sequencing technologies for rapid, high throughput analysis of the genome, transcriptome and epigenome [11]. These technologies provide the opportunity to identify prognostic markers and candidate therapeutic targets, advancing efforts to develop targeted therapies.

The two main types of targeted drugs are monoclonal antibodies and small molecule inhibitors. Many of these compounds have already been approved by the US Food and Drug Administration (FDA) to treat several types of cancers, including leukaemia, lymphoma, and cancers of the brain, thyroid, lung, breast, stomach, intestine, pancreas, liver, kidney, ovary, prostate, bone and skin [12, 13]. The most common targets include growth factor

receptors, signalling molecules, cell-cycle proteins, modulators of apoptosis and molecules involved in invasion and angiogenesis [14]. Unfortunately, although improvements in progression-free survival and life quality of treated patients have been observed in numerous clinical studies using these drugs, overall survival has not been prolonged because of later-acquired drug resistance [15]. One particularly challenging concept is that cancer is not a static entity and that many tumours potentially undergo continual genetic evolution, allowing adaptation to new selective pressures such as anticancer treatment [16, 17]. Tumour evolution and therapeutic failure are fostered by intratumoural heterogeneity, which can arise in multiple ways [18]. The most well-established mechanism involves intrinsic differences among cancer cells caused by stochastic genetic [19] or epigenetic [20] changes. Differences can also arise among cancer cells through extrinsic mechanisms in which different microenvironments within a tumour cause changes in cancer cell properties [21, 22]. Since the concept of cancer stem cells (CSCs) was introduced in late 1990s [23, 24], it has become clear that these long-lived and self-renewing cells may also be responsible for tumour heterogeneity and escape treatment (Figure 1A). A CSC could hypothetically originate from a stem, a progenitor or a differentiated cell. Cancer can then progress as a stem cell disease creating a hierarchical organization, in which a minority of tumourigenic cells give rise to phenotypically diverse non-tumourigenic cells. Alternatively, cancer can progress by clonal evolution of the tumour CSCs [25]. Moreover, the recently proposed CSC plasticity model suggests that these cell populations are dynamic and both CSCs and non-CSCs are capable of interconversion in response to environmental cues [26–28].

Although often considered as mutually exclusive models to describe tumour heterogeneity, the stochastic and CSC models of cancer can be harmonized by considering the role of genetic diversity and non-genetic influences in contributing to tumour heterogeneity [29, 30]. A tumour does not have one single tumour genome, but instead comprises multiple subclones bearing distinct genetic makeups, each with differing abilities to survive drug treatment. These subclones may evolve in parallel over the lifetime of a cancer and contribute to intratumoural heterogeneity. However, even within unique genetic subclones, not all cells function equally. Some cells retain capacity for self-renewal and long term clonal maintenance, some lay dormant and some fuel tumour growth, while most tumour cells are post-mitotic and destined for clearance [30]. This intratumour heterogeneity can lead to underestimation of the tumour genomics landscape portrayed from single tumour biopsy samples and may present major challenges to personalized medicine and biomarker development. Indeed, drug target mutations which are detected in some but not all biopsies for a single patient will result in obvious implications regarding the efficacy of any customized treatment plan [31]. By analyzing multiple samples from four patients with metastatic renal cell carcinoma that were taken before and after cytoreductive surgery, Gerlinger *et al*. found that about two thirds of the mutations found in single biopsies were not uniformly detectable throughout all the sampled regions of the same patient's tumour [32]. Intratumour genetic differences occurred in genes encoding proteins that are targets for some of the available anticancer drugs such as mTOR which will be described in the next paragraph.

mTOR signalling and cancer biology

The serine/threonine kinase mTOR integrates a wide variety of cellular signals, including mitogen and nutrient signals, to control cell proliferation and cell size [33]. mTOR can exist in at least two complexes differing in their subunit composition and sensitivity to rapamycin. mTOR complex 1 (mTORC1), is composed of mTOR, regulatory associated protein of mTOR (Raptor), mLST8/G-protein β-subunit like protein (GβL), RAS40 and Deptor. mTOR complex 2 (mTORC2), is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mLST8/GβL, stress-activated-protein-kinase-interacting protein 1 (Sin1), proline-rich repeat protein-5 (PRR-5)/protein observed with Rictor-1 (Protor-1), and Deptor. mTORC1 is highly sensitive to rapamycin, whereas mTORC2 is relatively insensitive.

mTOR complexes are differentially regulated by distinct upstream signals. Upstream of mTOR is the phosphatidylinositol 3-kinase (PI3K) pathway, a family of lipid kinases. In response to receptor-mediated survival signals, PI3K generates phospholipids, which in turn activate the serine/threonine kinase AKT and other downstream effector pathways [34]. Phosphatase and tensin homologue (PTEN) functions as the main negative regulator of PI3K signalling by dephosphorylating newly synthesized PI3K lipids and thus hampering AKT activation [35]. Once activated, AKT phosphorylates and inhibits tuberous sclerosis complex 2 (TSC2, also known as tuberin), disrupting its interaction with tuberous sclerosis complex 1 (TSC1, also known as hamartin) [36]. TSC1/TSC2 heterodimer functions as a GTPase activating protein for Ras homologue enriched in brain (Rheb), causing hydrolysis of Rheb-bound GTP to GDP [37]. The active GTP-bound form of Rheb directly interacts with mTORC1 to stimulate its activity [38]. Thus, AKT phosphorylation of TSC2 relieves the inhibitory effects of the TSC1/TSC2 complex on Rheb and mTORC1. Apart from AKT, AMP-activated protein kinase (AMPK) is a major regulator of mTORC1 [39]. AMPK is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [40]. Activation of AMPK by increased

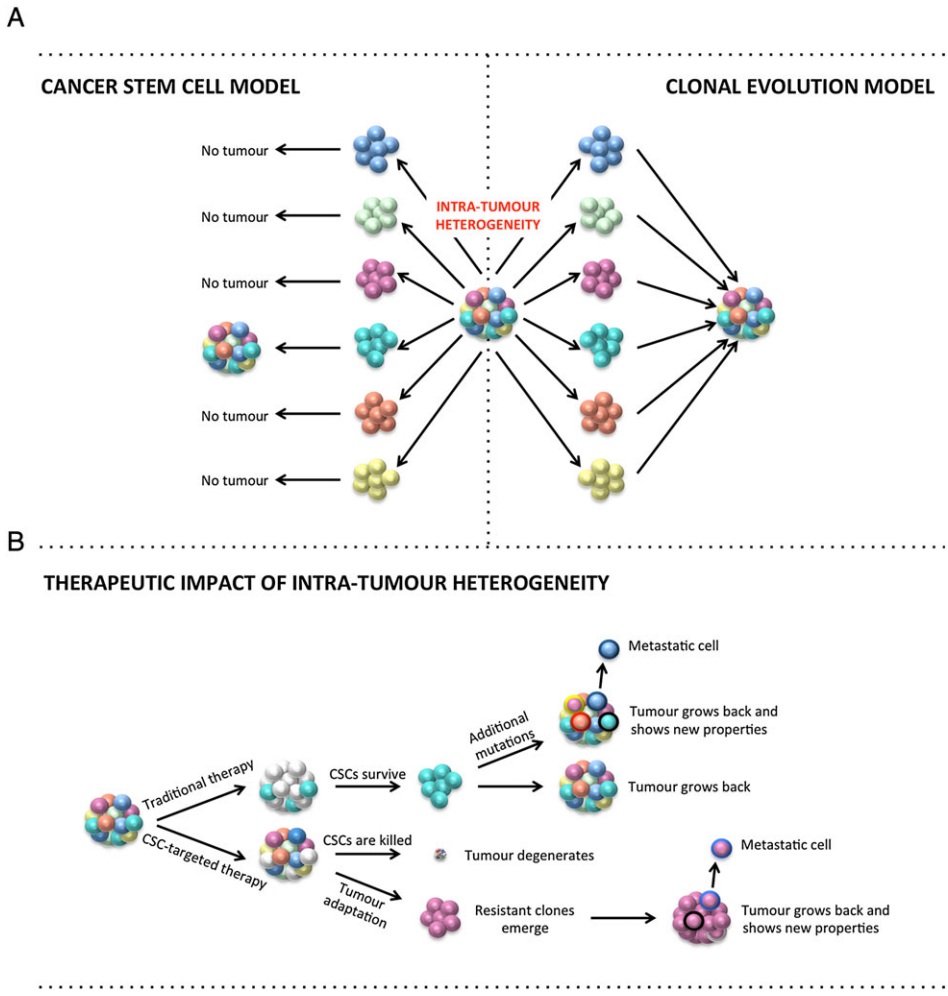

Figure 1

Schematic view of cancer stem cell (CSC) evolution during the course of the disease. A) Intra-tumour heterogeneity can arise from intrinsic differences between CSCs and their progeny or from clonal evolution. In the CSC model, only the CSCs have the ability to generate a tumour. In the clonal evolution model, every cell within a tumour has similar tumourigenic potential. The CSC hierarchical model suggests that CSCs are the only relevant target for therapy. In contrast, the clonal evolution model suggests that all tumour cells must be targeted, as all are equally able of causing relapse after therapy. **B**) While traditional therapy may initially control disease by effectively debulking tumours, the tumours invariably recur due to the ability of CSCs to survive and repopulate the tumour mass. However, the CSC model and the clonal evolution model are not mutually exclusive, and clonal evolution can happen in CSCs, leading to the acquisition of new tumour cell properties. CSC-targeted therapy renders tumours unable to maintain themselves or grow. However, resistant clones can emerge under the selective pressures of targeted therapy, leading to the emergence of a tumour with new characteristics

energy demands promotes decreased mTORC1 activity [41] in a TSC2- [42] or Raptor- [43] dependent pathway.

Amongst mTORC1 targets are ribosomal S6 protein kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), mediators of protein translation and cell growth [44]. mTOR's response to a wide range of intracellular (energy and stress) and extracellular (nutrients, growth factors, hormones) signals is mediated through these effectors. In response to nutrient and growth factor availability, mTORC1 suppresses autophagy, a self-degradative process by which metabolically stressed cells recycle cytoplasmic components to recover energy necessary for their survival [45]. mTORC1 also orchestrates anabolic cell growth by stimulating nucleotide synthesis through the pyrimidine synthesis pathway [46].

In contrast to mTORC1, the upstream regulation of mTORC2 is less well defined, although ribosome association has been classically considered a major, if not the sole, mechanism of mTORC2 activation [47]. However, very recently, PtdIns(3,4,5)P_3, which is generated upon insulin or growth factor stimulation and PI3K activation, has been identified as a direct upstream activator of mTORC2. Specifically, the pleckstrin homology (PH) domain of Sin1 interacts with the mTOR kinase domain (KD) to suppress mTOR activity. PtdIns(3,4,5)P_3 interacts with Sin1-PH to release its inhibition on mTOR-KD, thereby triggering mTORC2 activation [48].

mTORC2 plays an important role in cell survival, metabolism, proliferation and cytoskeleton organization through phosphorylation of protein kinase Cα (PKCα),

serum/glucocorticoid-regulated kinase 1 (SGK1), as well as AKT, thereby allowing for complete activation of AKT [49–52]. AKT is therefore both an upstream activator of mTORC1 and downstream effector of mTORC2.

Until recently, very few mutations had been found in the *MTOR* gene. It was generally believed that dysregulation of upstream pathways was mostly responsible for constitutive activation of the mTOR pathway. Indeed, many components of the PI3K signalling pathway are mutated in human cancers [53]. However, a diverse set of cancer-associated *MTOR* mutations conferring pathway hyperactivation was recently reported. Specifically, using publicly available tumour genome sequencing data, Grabiner *et al*. generated a comprehensive catalogue of mTOR pathway mutations in cancer, identifying 33 *MTOR* mutations [54]. The mutations clustered in six distinct regions in the C-terminal half of mTOR and occurred in multiple cancer types, with one cluster particularly prominent in kidney cancer [54]. Thus, screening for alterations in the mTOR pathway may help to identify subsets of patients who may benefit from targeted therapies directed against mTOR.

The mTOR paradox: opposing effects on normal and malignant stem cells

While mTOR activation contributes to cancer progression, and possibly also initiation, the prolonged stimulation of mTOR in normal cells can lead to stem cell depletion, reduced health and lifespan. This effect has been referred to as the'mTOR paradox' [55]. The most abundant information comes from studies of haematopoietic stem cells (HSCs) isolated from the bone marrow or blood. Constitutively active AKT depletes HSCs and induces leukemia in mice [56]. Similarly, *PTEN* deletion from HSCs not only leads to rapid HSC proliferation and depletion, but also changes HSC differentiation characteristics, allowing leukemia to develop [57]. These effects are mostly mediated by mTOR, as they can be inhibited by the first generation mTOR inhibitor rapamycin [58]. Rapamycin not only depletes leukemia-initiating cells but also restores normal HSC function [58]. Further proof of mTOR involvement in the maintenance of HSCs comes from a study showing rapid HSC cycling and depletion following *TSC1* deletion [59]. Consistently, in old mice, rapamycin delays mouse HSC ageing by preserving adult HSC self-renewal and haematopoietic capacity [60].

mTORC1 also regulates stem cell self-renewal in other systems. For example, excessive mTOR signalling leads to adult epidermal stem cell exhaustion and progressive hair loss in mice, a phenomenon that rapamycin can delay [61]. Additionally, caloric restriction increases intestinal stem cell numbers through mTORC1 inhibition [62]. Interestingly, not only does rapamycin treatment prevent stem cell exhaustion, but also extends longevity in old mice [63], indicating a crucial role of mTOR signalling in the regulation of lifespan.

Overall, these findings demonstrate the feasibility of perturbing the mTOR pathway to prevent tumour growth without disrupting the function of normal tissues and cells.

mTOR-targeted therapy: pitfalls and successes

Several classes of mTOR-targeted drugs are currently undergoing clinical trial evaluation for various cancers, yet success rates in bringing these drugs to the bedside remain low. Rapamycin and its derivatives (rapalogues) have proven ineffective in most clinical trials. The rapalogues, substrate-selective mTORC1 inhibitors, often fail due to an incomplete inhibition of mTORC1. Moreover, rapalogues do not inhibit mTORC2, although they can interfere with the assembly of mTORC2 complex in certain cell types [64]. As a consequence, rapalogues are unable to prevent mTORC2-mediated AKT activation. In addition, increased AKT S473 phosphorylation after rapalogue treatment may occur as the result of the suppression of S6K1-mediated negative feedback loops, which attenuate signalling via insulin/insulin-like growth factor-1 receptor (IGF-1R) and other tyrosine kinase receptors [65]. Suppression of these feedback loops unleashes over-activation of upstream pathways including PI3K, AKT and extracellular signal-regulated kinase (ERK), which counterbalance the antiproliferative effects of mTOR inhibitors and lead to drug resistance [66].

The recognition that rapalogues have limited substrate-specific efficacy and cause feedback activation of several oncogenic pathways has fuelled the development of dual PI3K/mTOR inhibitors to avoid PI3K pathway reactivation [67] and ATP-competitive mTOR kinase inhibitors (mTorKIs) [68]. mTorKIs cause more potent and durable inhibition of mTORC1 than rapalogues, and interfere with both mTORC1 and mTORC2 [69]. Of note, newly developed inhibitors have been shown to possess a stronger preferential inhibitory effect on CSCs than bulk tumour cells, thus providing a compelling rationale for the continued development of more specific and selective inhibitors for the treatment of cancer [70]. The following is a description of the most relevant studies investigating the therapeutic efficacy of mTOR inhibitors against CSCs.

Glioblastoma

Hyperactivation of the PI3K/AKT/mTOR pathway and inactivation of wild-type p53 by MDM2 over-expression are frequent molecular events in gliobastoma (GBM). Accordingly, a combined therapy utilizing the mTOR/AKT inhibitor FC85 and the MDM2/p53 inhibitor ISA27 produced a synergistic effect on the inhibition of GBM

cell viability as well as on the reactivation of the p53 pathway [71]. Most importantly, similar synergistic effects were shown in GBM-derived CSCs, where the simultaneous use of the two compounds induced strong differentiation as well as apoptosis [71].

Breast cancer

Activation of the mTOR pathway in breast cancer stem-like cells is required for colony formation ability *in vitro* and tumourigenicity *in vivo* [72]. In patient-derived mammary CSCs, tamoxifen, an antagonist of the oestrogen receptor, activated mRNA translation and ribosome synthesis via mTOR, thus accounting for therapeutic resistance and possibly tumour relapse [73]. This process could be successfully antagonized by mTOR inhibitors [73].

Pancreatic cancer

The first generation mTOR inhibitors have failed to show clinical efficiency in treating pancreatic cancers due in part to the feedback relief of the IGF-1R/AKT signalling pathway [74]. The second generation mTOR inhibitors, such as AZD8055, could inhibit AKT activation upon mTORC2 inhibition [75]. However, AZD8055 induced a temporal inhibition of AKT kinase activities and AKT was then rephosphorylated [75]. Additionally, AZD8055-induced transient AKT inhibition increased the expression and activation of epidermal growth factor receptor (EGFR) by releasing its transcriptional factors Fork-head box O 1/3a (FoxO1/3a) [75]. The combination of AZD8055 and the EGFR tyrosine kinase inhibitor erlotinib synergistically inhibited the mTORC1/C2 signalling pathway, EGFR/AKT feedback activation and cell growth, as well as suppressed the progression of pancreatic cancer in a xenograft model [75]. The mTOR pathway was additionally shown to maintain the stem cell-like properties of pancreatic cancer cells. Sphere formation under stem cell culture conditions and anchorage-independent colony formation were both dependent on this pathway. Consistently, inhibition of the pathway by rapamycin effectively reduced the viability of cancer stem-like cells [5].

Colon cancer

In our study, colon CSCs (CoCSC) exhibited strong mTORC2 expression, and rare expression of mTORC1 [76]. This latter correlated with tumour differentiation, being expressed in CoCSC-derived xenografts. We compared the effects of various mTorKIs (Ku-0063794, WYE-354, pp242, and Torin-1) with first-generation mTOR inhibitors (rapamycin and temsirolimus) on three CoCSC lines. mTOR inhibitors affected CoCSCs in a variety of ways, resulting in proliferation, induction of autophagy or apoptosis. The apoptosis-inducing mTOR inhibitor Torin-1 hindered growth, motility, invasion and survival of $CD326^+/CD24^+/CD49f^+/CD29^+$ and $CD326^+/CD44^+/CD166^+$ colon cancer cell subpopulations *in vitro*, and suppressed tumour growth *in vivo* with a concomitant reduction in vessel formation. Torin-1 also affected the expression of markers for cell proliferation, angiolymphogenesis and stemness *in vivo*. Our study also indicated that although Torin-1 resistant clones can emerge, they are poorly tumourigenic, thus encouraging its potential use for colon cancer therapy. Finally, through an innovative system based on the use of the mouse lymph node as an *in vivo* bioreactor [77], we showed that Torin-1 does not affect the survival of normal colon stem cells *in vivo*, supporting other observations of mTorKI selectivity towards CSCs.

Prostate cancer

Putative prostate CSCs exhibited high PI3K/mTOR pathway activity and treatment with the dual PI3K/mTOR inhibitor BEZ235 suppressed their proliferation [78]. Similarly, prostate cancer radioresistance was associated with epithelial–mesenchymal transition (EMT) and enhanced CSC phenotypes via activation of the PI3K/Akt/mTOR signalling pathway [79]. The combination of BEZ235 with radiotherapy was shown as a promising modality to overcome radioresistance in the treatment of prostate cancer [79].

Ovarian cancer

By targeting the mitochondria, the isoflavone derivative NV-128 promoted caspase independent cell death of rapamycin-resistant ovarian CSCs through the mTOR pathway [80]. Specifically, NV-128 activated two independent cell death pathways. Degradation of cytochrome C oxidase (COX) subunit IV led to ATP loss and increase of mitochondrial reactive oxygen species (ROS). ATP loss was in turn able to create a depleted energy status, which activated the energy sensing AMPK leading to inhibition of mTOR. This inhibitory effect was sufficiently potent to induce autophagic cell death in the ovarian CSCs. ROS activated the ERK/Bax axis leading to loss of mitochondrial membrane potential and endonuclease G-dependent DNA fragmentation.

Overall, these studies demonstrate that the mTOR axis can be a promising target in treatment protocols for different types of cancer.

Conclusions

Current first line chemotherapy generally consists of cytotoxic agents. While these agents may initially control disease by effectively debulking tumours, the tumours invariably recur due to the ability of CSCs to survive and repopulate the tumour mass (Figure 1B) [81]. Intensive effort is currently devoted to targeting the mTOR pathway due to its emerging role in the maintenance of CSCs. Unfortunately, although there are a high number of

mTOR inhibitors available, the selection of a successful regimen that maximally suppresses tumour progression continues to be challenging. Because most tumours are heterogeneous, a single drug regimen for patients with the same histologic tumour type is not always appropriate. Considering this, it is imperative to identify which cancer patients may benefit from mTOR inhibitor therapy. *MTOR* mutations may be used to classify better patients. Nevertheless, the presence of *MTOR* mutations in a tumour does not necessarily imply that the tumour was actually driven by the mutated mTORs or that this tumour will be clinically responsive to mTOR-targeted therapies. Acquired resistance to targeted therapies is another challenge to consider (Figure 1B). Unravelling the interactions of drugs and genetics will, in the long run, facilitate the rise of personalized medical practices.

Competing Interests

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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