

Molecular Pathways: Anticancer Activity by Inhibition of Nucleocytoplasmic Shuttling

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Abstract

A dynamic distribution between nucleus and cytoplasm (nucleocytoplasmic shuttling) is one of the control mechanisms adapted by normal cells to regulate the activity of a variety of molecules. Growing evidence suggests that dysregulation of the nucleocytoplasmic shuttling is involved in promoting abnormal cell survival, tumor progression, and drug resistance, and is associated with poor cancer prognosis. Aberrant nucleocytoplasmic shuttling in cancer cells may result from a hyperactive status of diverse signal-transduction pathways, such as the PI3K–AKT and MAPK pathways, or from alterations in the general nuclear import/export machinery. Among the large number of molecules

involved in the shuttling process, exportin XPO1, also known as chromosome region maintenance 1, appears to play a particularly prominent role in pathogenesis of both hematological malignancies and solid tumors. Given the importance of nucleocytoplasmic shuttling in cancer pathogenesis and the rapidly expanding knowledge in this field, attempts have been made to develop compounds able to revert the aberrant nucleocytoplasmic shuttling. A promising new drug, KPT-330 (Selinexor), which belongs to the class of XPO1 inhibitors called selective inhibitors of nuclear export, is now being tested in phase I/II clinical trials. *Clin Cancer Res*; 21(20); 4508–13. ©2015 AACR.

Background

Physical separation of the nucleus from the cytoplasm by the nuclear envelope is a hallmark of eukaryotic cells. The proper spatiotemporal localization of molecules in these two compartments is crucial for cellular homeostasis, and is regulated by a bidirectional transport system channeled through the nuclear pore complex (NPC). NPC provides a selective portal for movement across the nuclear envelope: small molecules (<40 kDa) can passively diffuse across the NPC, whereas the transport of larger molecules, including most proteins and RNAs, is a receptor- and energy-dependent process (1).

Nucleocytoplasmic transport receptors are termed karyopherins, a family of 20 proteins that mediate the shuttling of proteins from cytoplasm to nucleus (importins) or from nucleus to cytoplasm (exportins) by recognizing specific transport signals in the cargo proteins (2; Fig. 1). The best characterized nucleocytoplasmic transport signals include the classical nuclear localization signal (NLS), required for importin-mediated entry into the nucleus, and the leucine-rich nuclear export signal (NES), required for exportin-mediated exit from the nucleus (3, 4). The Ras-related nuclear protein small GTPase confers directionality to the transport process by regulating cargo loading and unloading by the karyopherins (5).

Nucleocytoplasmic shuttling dysregulation in cancer

A dynamic subcellular compartmentalization via nucleocytoplasmic shuttling is one of the regulatory mechanisms used by normal cells to modulate the activity of a variety of molecules. Mislocalization of those molecules may alter their activities, thus disturbing the homeostasis of the cells and causing diseases such as cancer. Growing evidence suggests that dysregulation of nucleocytoplasmic shuttling is involved in promoting cancer cell survival, carcinogenesis, tumor progression, and drug resistance (6). Mislocalization of tumor suppressor proteins (TSP) appears to play a key role in cancer pathogenesis. Given that many TSPs execute their antineoplastic functions within the nucleus, mechanisms that enhance their nuclear export and/or cytoplasmic sequestration effectively result in their functional inactivation (7). Likewise, there is also evidence that the activity of oncoproteins can be influenced by their subcellular localization. All these can result from alterations in the shuttling machinery, which is frequently detected in cancer.

Oncogenic signaling pathways and dysregulated nucleocytoplasmic shuttling of TSPs

Posttranslational modifications of the NLS or NES motifs in the cargoes, such as phosphorylation, methylation, and ubiquitylation, can modulate binding affinity of the cargoes to specific karyopherins, thus affecting nucleocytoplasmic shuttling (1). Signaling pathways, such as PI3K–AKT and MAPK, are known to play roles in such modifications (8), and aberrant activation of these pathways can lead to mislocalization and functional alterations of several TSPs, including cyclin–dependent kinase inhibitor 1A (CDKN1A and p21^{Cip1}), CDK inhibitor 1B (CDKN1B and p27^{Kip1}), forkhead box O (FOXO) proteins, and TP53 (7).

The CDKN1A and CDKN1B act as tumor suppressors in the nucleus through inhibition of cyclin–dependent kinases (CDK) during cell-cycle progression (9, 10). However, they may acquire

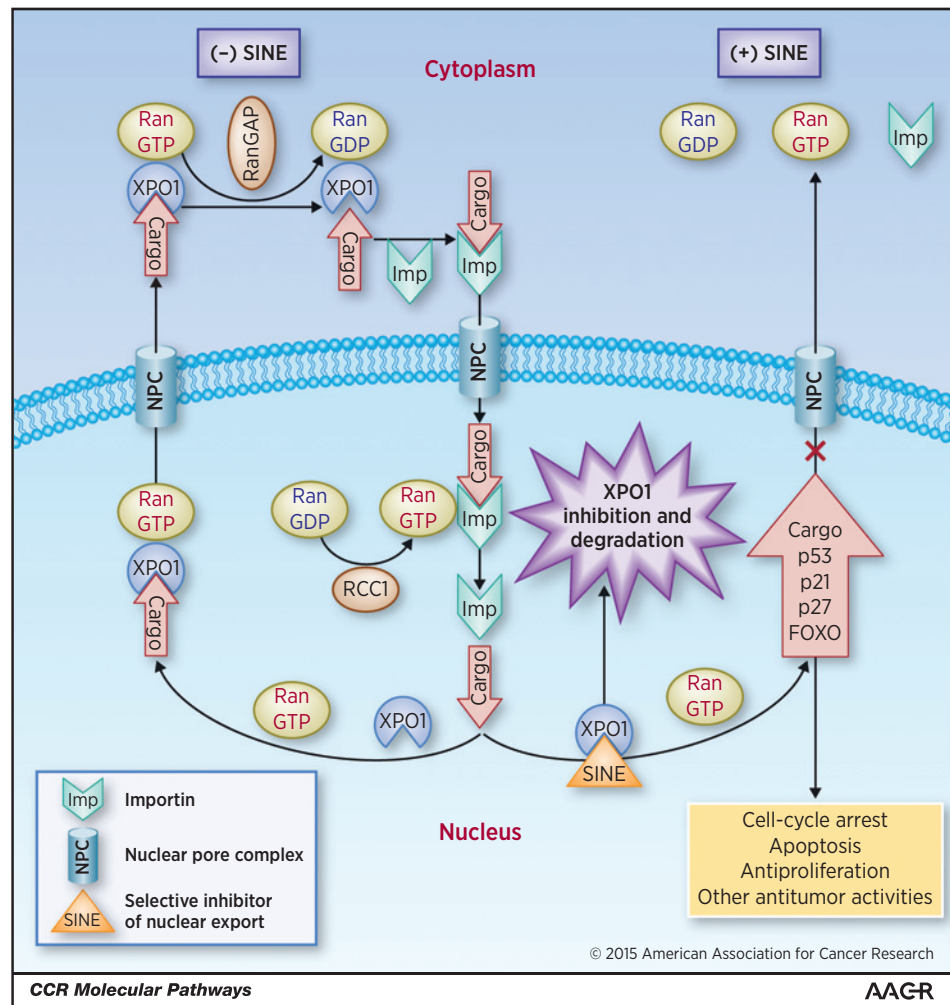
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Figure 1. Diagram of nucleocytoplasmic shuttling and the effects of XPO1 inhibition. In the cytoplasm, importin (Imp) forms a complex with cargo protein by recognizing its NLS. The complex passes through the nuclear pore complex (NPC) into the nucleus, where cargo is released upon binding of RanGTP to importin. In the nucleus, XPO1 binds to cargo by recognizing the NES, and together with RanGTP, is exported into the cytoplasm. Following the conversion of RanGTP to RanGDP catalyzed by Ran-GTPase-activating protein (RanGAP), the cargo is dissociated from the complex and released into the cytoplasm. However, in the presence of SINE, XPO1 is inhibited and degraded, and is unable to export its cargo proteins. This leads to nuclear accumulation of important TSPs, including p53, p21, p27, and FOXO, resulting in cell-cycle arrest, apoptosis, antiproliferation, and other antitumor activities. RCC1 is a guanine nucleotide exchange factor for RanGTPase, and guides the exchange of RanGDP to RanGTP in the nucleus.



oncogenic properties when mislocalized in the cytoplasm, leading to increased cell migration and invasion through the inhibition of Rho proteins and their effector Rho-kinase (11, 12). Phosphorylation of CDKN1A by AKT and PKC inhibits CDKN1A nuclear import, whereas CDKN1B phosphorylation by AKT and ERK enhances CDKN1B nuclear export, thereby contributing to their inappropriate cytoplasmic localization (10–12). Such mislocalization has been observed in esophageal, thyroid, colon, breast, and ovarian cancers, and is related to higher histologic grade, advanced stage of disease, and poorer patient survival (9–12).

The FOXO transcription factor family (FOXO1a, FOXO3a, FOXO4, and FOXO6) represent one of the most relevant targets downstream of the PI3K–AKT pathway, whose hyperactivity leads to inhibition of apoptosis and induction of cell proliferation (13). When localized in the nucleus, FOXO proteins act as tumor suppressors by upregulating the inhibitors of cell proliferation (CDKN1B and RB family member p130), and of survival (BIM, Fas ligand, and TRAIL; ref. 14). FOXO proteins are inactivated by AKT-mediated phosphorylation that promotes their interaction with exportin XPO1 and facilitates FOXO export to the cytoplasm. (15, 16). It has been shown that prostate and renal cancer cell lines with a deficiency of PTEN (a negative regulator of the PI3K–AKT

pathway) display a constitutive cytoplasmic mislocalization of inactive FOXO1a (17). Importantly, reconstitution of FOXO1a nuclear localization restores its transcriptional activity in PTEN-null cells, leading to cell-cycle arrest and apoptosis (17). Also, forced expression of nuclear FOXO proteins has been shown to induce apoptosis in a wide range of cancer cells, including breast cancer and malignant melanoma, although it could also exert paradoxical oncogenic effects in specific tumor histotypes and genetic context (18–24).

In human cancer, TP53 is the most frequently inactivated tumor suppressor and nearly half of the cancer cases harbor its loss-of-function mutations or deletions (25). Inactivation of TP53 can also occur due to aberrant nuclear exclusion of the wild-type protein (26). Indeed, abnormal cytoplasmic overexpression together with lack of nuclear presence of wild-type TP53 have been observed in many tumor types, including inflammatory breast carcinomas, neuroblastomas, retinoblastomas, colorectal, and ovarian cancers (26–30). Data from human neuroblastoma cell lines show that cytoplasmic entrapment of wild-type TP53 is sufficient to cause the loss of TP53-mediated cell-cycle arrest induced by DNA-damaging agents. In these cell lines, restoration of TP53 nuclear localization results in the recovery of its tumor suppressor function (31). Several potential mechanisms

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Table 1. Selected tumor suppressor proteins that are exported from the nucleus by XPO1, and their roles in cancer

Protein	Tumor suppressor activity	Implicated cancer
APC	Inhibitor of the Wnt signaling pathway through degradation of β -catenin	Colorectal cancer
BRCA1	Critical roles in DNA repair, cell-cycle checkpoint control, and maintenance of genomic stability	Breast cancer
p21 ^{CIP1}	Involved in cell-cycle arrest in the G ₁ phase in the presence of DNA damage	Breast, ovarian cancer, CML
p27 ^{KIP1}	Blocks cell cycle in the G ₀ -G ₁ phase upon differentiation signals or cellular stress	Thyroid, colon, ovarian, esophageal, breast carcinomas
FOXO1, 3a, 4, and 6	Induce expression of target genes with antiproliferative and proapoptotic activities	Breast, prostate, and thyroid cancer, glioblastoma, melanoma
INI1/SNF5	Subunit of the SWI/SNF ATP-dependent chromatin-remodeling complex that relieves repressive chromatin structure	Malignant and atypical teratoid rhabdoid tumors
I κ B α	Inhibitor of pro-survival NF κ B transcription factor by sequestering it in the cytoplasm	Multiple myeloma, C-ALL, Hodgkin disease, breast, ovary, colon, pancreas, thyroid carcinoma
Merlin/NF2	Membrane cytoskeleton protein with a role in the regulation of contact-dependent inhibition of proliferation and in the transmembrane signaling	Neurofibromatosis type 2
NPM1	Nucleolar protein implicated in ribosomal protein assembly and transport, control of centrosome duplication, and regulation of the tumor suppressor ARF	AML, breast cancer
p53	Regulates target genes that induce cell-cycle arrest and DNA repair, apoptosis, and senescence in response to DNA damage	Colorectal, ovarian and inflammatory breast cancer, RB, neuroblastoma
RASSF2	Interacts with KRAS and controls cell growth and survival	Thyroid cancer, nasopharyngeal carcinoma
RB	Inhibits cell-cycle progression from G ₁ -S-phase by targeting the E2F transcription factors	RB, small cell lung cancer, cervical cancer, AIDS-related Burkitt's lymphoma
RUNX3	Functions as a tumor suppressor by attenuating Wnt signaling and enhancing TGF β signaling	Colon, gastric, hepatocellular, breast, pancreatic, prostate, laryngeal carcinomas
TOB	Member of the TOB/BTG antiproliferative (APRO) protein family	Breast, lung, thyroid cancer
VDUPI	Upregulated by various stresses (irradiation, heat shock, serum starvation, and TGF β), eventually leads to the inhibition of cell proliferation	Breast, lung, colon, cervical cancer
Wee1	Induces G ₂ cell-cycle arrest through the inhibition of CDK1, allowing the premitotic DNA repair	Breast, lung, colon cancer

Abbreviations: AML, acute myeloid leukemia; C-ALL, childhood acute lymphoblastic leukemia; CML, chronic myeloid leukemia; RB, retinoblastoma.

underlying cytoplasmic sequestration of TP53 have been described. These include increased nuclear export due to overexpression or hyperactivation of MDM2, mutations in the TP53 NLSs, and overexpression of cytoplasmic proteins able to bind and trap TP53, such as the glucocorticoid receptor and the parkin-like ubiquitin ligase protein (27, 32, 33). Although TP53 nuclear export is facilitated by the nuclear export receptor exportin-1 (XPO1), TP53, on the other hand, represses XPO1 expression in response to DNA damage in normal cells (34). Overexpression of XPO1 in cancer cells may disrupt this feedback regulatory loop, leading to overly decrease of nuclear TP53 concentration and inadequate DNA damage response.

Alterations of nuclear export receptor XPO1 in tumors

Dysregulated nucleocytoplasmic shuttling in tumors can also be caused by an alteration in the transport machinery. A large number of molecules are involved in the shuttling process; nevertheless, alteration of the exportin XPO1, also known as chromosome region maintenance 1, plays a particularly prominent role in tumor pathogenesis. XPO1 is one of seven exportins expressed in mammalian cells, and it exports approximately 220 different proteins as well as a small subset of RNAs from nucleus to the cytoplasm. Interestingly, XPO1 is the sole nuclear export receptor for a large number of TSPs (see Table 1), with a key role in the control of several cancer-related processes, including cell-cycle progression, apoptosis, metastasis, and drug resistance (1).

Overexpression of XPO1 has been observed in many types of human solid tumors and hematologic malignancies (35–38). Importantly, XPO1 overexpression has usually been correlated with higher tumor grade, more advanced tumor stage, and poor prognosis (34, 37, 39), suggesting its involvement in tumorigenesis. Exogenous overexpression of XPO1 resulted in the transformation of human normal bronchial epithelial cells, whereas its inhibition significantly delayed the growth of A549 NSCLC xenograft tumors in mice (39). Recent experimental data also support a possible role of XPO1 in carcinogen-induced lung cancer development (39). Moreover, whole-genome sequencing analysis revealed somatic mutations in XPO1 in approximately 4% of patients with chronic lymphocytic leukemia (CLL), all affecting the same glutamic residue (E571; ref. 40). The recurrent nature of the E571 mutation suggests that it may represent an "oncogenic driver," with a causative role in CLL leukemogenesis. A different missense mutation (D624G) in XPO1 has also been identified in 1 patient with esophageal squamous cell carcinoma (41). Nevertheless, the pathologic role of these XPO1 mutations and the underlying molecular mechanism remain to be elucidated.

Clinical-Translational Advances

Given the critical role of XPO1 in nucleocytoplasmic shuttling and in tumor pathogenesis, its inhibition has emerged as a therapeutic strategy in cancer. The rationale behind the targeting of XPO1 is to increase the nuclear concentration of

XPO1 cargoes, in particular of tumor suppressor gene products. It must be noted, however, that XPO1 also plays a role in RNA export and in mitotic processes, such as microtubule nucleation at kinetochores (42). Thus, interference with these XPO1 functions may also contribute to the therapeutic effect of XPO1 inhibition.

The first XPO1 inhibitor discovered was Leptomycin B (LMB), a natural compound isolated from the bacteria *Streptomyces* species AT51287 (43). It is an irreversible inhibitor that covalently binds a cysteine residue (C528) in the cargo-binding region of XPO1, preventing cargo interaction with XPO1 (43). LMB has potent antitumor activity *in vitro*; however, it only induced a transient reduction of tumor biomarkers, such as cancer antigen 125 (CA-125), and human chorionic gonadotropin in patients with ovarian carcinoma and trophoblastic tumor, respectively, and one stable disease in a sarcoma patient in a phase I trial (44). The severe toxicity profile of LMB has prevented its further clinical development (44). Toxicities have been attributed to off-target effects due to its binding to several cysteine proteases, in addition to the irreversible inhibition of XPO1 (45).

Subsequently, several natural products such as Ratjadone as well as synthetic compounds (KOS-2464, PKF050-638, and CBS9106) have been developed. All these compounds inhibit XPO1 by binding its C528 residue, causing cell-cycle arrest and apoptosis in a time- and dose-dependent manner in a broad spectrum of cancer cells. These inhibitors are more clinically relevant because they are much less toxic than LMB, while maintaining high potency (46–48).

The newer additions of XPO1 inhibitors are a group of small molecule compounds called selective inhibitors of nuclear export (SINE), including KPT-115, KPT-127, KPT-185, KPT-251, KPT-276, KPT-330 (Selinexor), and KPT-335 (Verdinexor). Unlike LMB, SINEs bind reversibly the C528 residue in XPO1, with virtually no off-target activity. KPT-330 (Selinexor) has an IC₅₀ of about 20 nmol/L for XPO1 inhibition, but has minimal activity (>10 μmol/L) against 114 other proteins, including enzymes, receptors, transporters, ion channels, and other cysteinyl-active site kinases and proteases (45).

SINE compounds have been shown to inhibit nuclear export of many TSPs with key roles in genomic stability and DNA repair (TP53, TP73, and BRCA1), cell-cycle control (pRB1, CDKN1A, and CDKN1B), and apoptosis [FOXO proteins, adenomatous polyposis coli (APC) protein, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα)] in cancer cell lines and tumor biopsies (49–56; Fig. 1). Notably, preclinical data suggest that the cytotoxic effect of XPO1 inhibitors in different tumor types may specifically rely on nuclear entrapment of certain XPO1 cargoes with particular biologic activities. For example, the activity of SINE compounds in some breast cancer cell lines seems to depend largely on the inhibition of survivin shuttling (49). Survivin, a member of the inhibitors of apoptosis protein family, is highly expressed in breast cancer cells, and its cytoplasmic levels have been shown to be an independent predictor of poor prognosis, whereas its nuclear levels were associated with a favorable prognosis (55). Survivin nuclear export exclusively relies on XPO1, and a significant depletion of cytoplasmic survivin can be induced by XPO1 inhibition. The proapoptotic activity of SINEs in breast cancer cell lines largely depends on inhibition of survivin shuttling, and restoring survivin levels by ectopic

overexpression is sufficient to impair the proapoptotic effect of SINEs (49). In TP53 wild-type NSCLC cells, however, the antiproliferative effects of SINEs mainly depends on the increase of nuclear TP53, because concomitant silencing of TP53 expression or its inhibition by pifithrin-α causes SINE resistance. Interestingly, XPO1 inhibition also displays a cytotoxic activity in a TP53 mutated lung cancer cell line. In this case, the activity of SINEs seems dependent on TP73, a member of the TP53 family, that is also involved in DNA damage-induced cell-cycle arrest and apoptosis and regulates TP53-dependent genes in TP53-deficient cells (50).

Altered nucleocytoplasmic shuttling may contribute to drug resistance as well, and in this regard, XPO1 inhibitors have shown synergistic anticancer activity when used in combination with chemotherapy or targeted therapeutic agents. Aberrant nuclear export of topoisomerase II is one of the mechanisms of resistance to doxorubicin and etoposide in myeloma cells, and such resistance can be reverted by concomitant inhibition of XPO1 (57). In chronic myeloid leukemia (CML) cells with t(9;22) chromosome translocation, LMB is able to revert their acquired resistance to imatinib that targets BCR-ABL1 fusion oncoprotein. ABL proto-oncogene 1, nonreceptor tyrosine kinase (ABL1) acts in the nucleus as a tumor suppressor in normal cells, whereas BCR-ABL1 in the CML cells is constantly exported by XPO1 to the cytoplasm to exert its mitogenic and antiapoptotic activities (57). Interestingly, when BCR-ABL1 protein is forced into the nucleus by XPO1 inhibition, it retains the proapoptotic functions like that of ABL kinase. Further, imatinib and LMB combination induces significant nuclear entrapment of BCR-ABL1, leading to the death of imatinib-resistant cells (57).

An interesting feature of the XPO1 inhibition by SINEs is that it has negligible toxicity in normal hematopoietic and epithelial cells with IC₅₀ more than 5 to 20 μmol/L. Continuous (~72 hour) exposure of non-neoplastic cells to SINEs at nanomolar concentrations only induces cell-cycle arrest without apoptosis (45, 53). The differential effect of SINEs on normal and neoplastic cells is not yet fully understood. However, one plausible explanation is that restoration of TSPs in the nucleus by XPO1 inhibition triggers the apoptotic pathways in response to extended DNA damage accumulated in the neoplastic cells.

So far, KPT-330 (Selinexor) is the only XPO1 inhibitor in the phase I/II clinical trials. Compared with LMB, Selinexor showed a much better toxicity profile. Most adverse events in patients with solid tumors and hematologic malignancies were reversible grade 1 and 2, primarily nausea, anorexia, and fatigue. Among 106 patients evaluable for response, an overall disease control rate of 49% with some partial responses were observed in colorectal, melanoma, ovarian, and cervical cancer (58–61). Preliminary results of an ongoing phase II clinical trial evaluating the activity of single-agent Selinexor in patients with heavily pretreated, progressive gynecologic cancers, showed promising anti-tumor activity across ovarian, endometrial, and cervical cancers. The disease control rate was up to 52% (33 of 63 patients), with several patients remaining on study for up to 12 months (61). Durable responses and disease stabilization with single-agent Selinexor were also observed in hematologic malignancies across all disease subtypes, with some patients remaining on study for over 1 year (59, 60). Currently, 36 clinical trials with Selinexor were registered at the ClinicalTrials.gov database (<http://clinicaltrials.gov/ct2/home>).

Conclusions

Nucleocytoplasmic shuttling has been identified to have a role in cancer pathogenesis, and this has led to the development of new therapeutic strategies to revert its alterations. Particularly, the inhibition of XPO1, leading to nuclear retention and functional reactivation of TSPs, is the most advanced therapeutic strategy. Numerous new drugs have been developed and among them, Selinexor is currently being evaluated in phase I/II human clinical trials with promising preliminary results in hematologic malignancies and solid cancers. Although interfering with nucleocytoplasmic transport machinery could be detrimental to all cells, the new XPO1 inhibitors have been shown to preferentially suppress or eliminate tumor cells, relatively sparing normal cells.

Despite significant progress, several crucial questions remain unresolved. Patient selection appears challenging, as we do not know which cargoes are important, and these may vary from tumor type to tumor type and even from patient to patient. Furthermore, therapeutic efficacy of XPO1 inhibitors is hampered by intrinsic and acquired resistance, as evidenced by the prelim-

inary results from phase I/II clinical trials. Elucidation of the resistant mechanisms will be necessary for the development of sound combination strategies. Nonetheless, growing data clearly show that targeting the nucleocytoplasmic shuttling is a worth strategy to pursue.

Disclosure of Potential Conflicts of Interest

G. Giaccone reports receiving a commercial research grant from Karyopharm Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: F. Conforti, Y. Wang

Development of methodology: F. Conforti

Writing, review, and/or revision of the manuscript: F. Conforti, Y. Wang, J.A. Rodriguez, A.T. Alberobello, Y.-W. Zhang, G. Giaccone

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