Natural phytochemicals as testable therapeutic alternatives for HER-2-enriched breast cancer (Review)

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Abstract. The human epidermal growth factor receptor-2 (HER-2)-enriched molecular subtype of breast cancer responds to HER-2 targeted and/or to endocrine therapy, depending on the presence of functional hormone receptors. These long-term therapeutic options are associated with systemic toxicity and acquired drug resistance. Resistance to conventional and targeted chemo-endocrine therapy leads to the emergence of drug-resistant cancer stem cells that promote therapy-resistant disease progression. Relatively non-toxic natural phytochemicals may provide testable alternatives to therapy-resistant breast cancer. The present review summarizes data on the following: i) Growth inhibitory efficacy of mechanistically distinct natural phytochemicals in a preclinical model for HER-2-enriched breast cancer; ii) drug-resistant stem cell model for HER-2-enriched breast cancer; and iii) proof of concept for efficacy of natural phytochemicals as testable alternatives against drug-resistant cancer stem cells. Relative to the non-tumorigenic human mammary epithelial 184-B5 cells, HER-2 expressing tumorigenic 184-B5/HER cells (HER-2-enriched breast cancer model) exhibit hyper-proliferation and increased anchorage independent colony formation. Resistance to lapatinib, a small molecule inhibitor of EGFR and HER-2, provides the LAP-R phenotype that exhibits increased tumor spheroid formation and an upregulated expression of the stem cell markers, CD44, NANOG and OCT-4. Select bioactive natural phytochemicals, such as cruciferous glucosinolate, tea polyphenol, soy isoflavone and rosemary terpenoid at their respective maximum cytostatic concentrations exert anti-proliferative and pro-apoptotic effects on parental 184-B5/HER cells and downregulate the phosphorylation of HER-2. The expression of stem cell markers in the LAP-R phenotype is effectively inhibited by a bioactive terpenoid. Collectively, these data validate an experimental approach to identify efficacious natural phytochemicals as testable therapeutic alternatives for chemo-endocrine therapy resistant breast cancer.

Contents

1. Introduction

Global gene expression profiling of clinical breast cancer has provided molecular classification of subtypes based of differential expression of genes for hormone and growth factor receptors (1). This molecular classification dictates specific conventional chemo-endocrine therapy or pathway selective small molecule inhibitor based targeted therapy. Thus, for the hormone receptor-positive/human epidermal growth factor receptor-2 (HER-2)-positive luminal B molecular subtype, treatment with selective estrogen receptor modulators, aromatase inhibitors and HER-2 inhibitors represent common options (2). By contrast, for the hormone receptor-negative/HER-2-positive HER-2 enriched subtype HER-2-targeted therapy represents a viable option (3).

Long-term treatment with conventional and/or targeted therapy is frequently associated with systemic toxicity and acquired tumor resistance that collectively compromise patient compliance and favor therapy-resistant tumor progression predominantly due to the emergence of drug-resistant cancer stem cells (4).

In preclinical investigations, human breast carcinoma-derived BT474, MDA-MB-361 and MCF-7/HER cell models for the luminal B subtype, and SKBr-3 and MDA-MB-435 cell models for the HER-2-enriched subtype (5,6), represent valuable experimental systems with which to elucidate the mechanisms of drug resistance and for the identification of efficacious lead compounds. Multiple drug resistance via

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acquired resistance to HER-2/EGFR-selective small molecule inhibitors in the HER-2-enriched model (3) or cross-resistance to aromatase inhibitors in the MCF-7^{AROM} model (7,8) represent additional approaches. Recent advances in the development of human cancer models include genetically engineered mouse models and constituent cells lines, patient-derived xenografts and constituent organoid models, and drug-resistant cancer stem cell models. Collectively, these approaches promise the clinical translation of preclinical data.

Acquired drug resistance leads to the lack of a response to conventional and/or targeted chemo-endocrine therapy, and is frequently is associated with therapy-resistant cancer stem cells that are characterized by pluripotency, tumor-initiating potential and the upregulated expression of several cell surface proteins and nuclear transcriptional factors (9).

The limitations of chemo-endocrine and/or targeted therapy emphasize an unmet need to develop clinically relevant cancer stem cell models and identify efficacious testable alternatives for stem cell targeted therapy of chemo-endocrine therapy resistant breast cancer.

The present review summarizes a comparison of recently generated unpublished data with that from previously published data on the following: i) Growth inhibitory efficacy of mechanistically distinct natural phytochemicals on a cellular model for the HER-2-enriched breast cancer subtype; ii) drug-resistant stem cell model for HER-2-enriched breast cancer; and iii) proof of concept for efficacy of natural phytochemicals as testable alternatives for drug resistant breast cancer stem cells.

2. Cellular models

The human tissue-derived cellular models included in the present review are distinct from those established human breast carcinoma-derived cell lines as models for the HER-2-enriched breast cancer subtype. In the present 184-B5/HER model, non-tumorigenic human mammary epithelial cells (10) stably transfected with HER-2 oncogene exhibit tumorigenic transformation due to the overexpression of the HER-2 oncogene (11).

Cell lines in experimental models. The following cell lines represent the experimental models: i) 184-B5: This cell line was established from a histologically normal reduction mammoplasty sample. These cells lack the expression of ER, PR and HER-2 and are non-tumorigenic (10). These cells represent a baseline control for investigations on 184-B5/HER and Lapatinib resistant (LAP-R) models. ii) 184-B5/HER: This cell line over-expresses HER-2 oncogene. The cells are ER/PR negative and HER-2 positive, and produce tumors on *in vivo* transplantation (11). iii) 184-B5/HER (LAP-R): These cells are resistant to lapatinib, a small molecule inhibitor of EGFR and HER-2. These cells exhibit the downregulated expression of p-EGFR and p-HER-2, and the upregulated expression of several stem cell specific cellular and molecular markers (12).

Quantitative end point biomarkers. The quantitative parameters include population doubling times, saturation density, as monitored by viable cell number using trypan blue dye exclusion assay. Cell cycle progression and cellular apoptosis are monitored by flow cytometry and presented as the

G_1:$S+G_2$/M ratio and the sub-G_0 phase. The expression levels of select cell cycle regulatory and apoptosis-specific proteins are monitored by flow cytometry-based quantitative immuno-fluorescence assay that involves the sorting of cells positive for FITC-conjugated antibody (13). The fluorescence data are expressed as log mean fluorescent units (FU) per 10^4 fluorescent events following normalization by FU of cells stained with FITC-IgG antibody.

The statistically significant differences between the control and treatment groups were analyzed using a two sample Student's t-test, one-way ANOVA and Dunnett's post hoc multiple comparisons test with a threshold of $\alpha=0.05$, and the Chi square test where appropriate.

3. Natural phytochemicals

These agents are selected based on their documented chemo-preventive efficacy in preclinical models of epithelial organ site cancers. To ascertain the feasibility of the experimental approach, select phytochemicals, such as cruciferous glucosinolate indole-3-carbinol (I3C), tea polyphenol epigallo-catechin gallate (EGCG), soy isoflavone genistein (GEN) and rosemary terpenoids carnosic acid (CA) and carnosol (CSOL), are tested on the non-tumorigenic 184-B5 cells. Subsequently these agents are tested for their growth inhibitory efficacy on the 184-B5/HER model. Compared to the 184-B5 model, the 184-B5/HER model exhibits substantially greater growth inhibitory efficacy. At the mechanistic levels, I3C alters the cellular metabolism of estradiol to generate the anti-proliferative metabolite 2-hydroxyestrone (14). EGCG and GEN inhibit the expression of proliferative cell nuclear antigen (PCNA) and cyclin D1, and increase the expression of the cyclin-dependent kinase inhibitor (CDKI) p16^{INK4} (15). CA and CSOL inhibit tyrosine kinase expression and upregulate p16^{INK4} expression (15), induce cytostatic G_2/M arrest and accumulate G_2-specific cyclin B1 (16). CSOL also inhibits the expression of inducible cyclo-oxygenase-2 (COX-2) via the protein kinase C/mitogen-activated protein kinase (PKC/MAPK) pathway (17).

4. Status of hyper-proliferation

The data on the status of hyper-proliferation in 184-B5/HER model are summarized in Table I. In comparison with the non-tumorigenic 184-B5 cells, the hyper-proliferative 184-B5/HER cells exhibit an approximately 55% reduction in population doubling times, an approximately 63% increase in saturation density, an approximately 96% reduction in the sub-G_0 (apoptotic) population and a substantial increase in anchorage-independent colony formation, the latter being an established *in vitro* surrogate end point for *in vivo* tumor development (16). In addition, the 184-B5/HER cells exhibit an approximately 178-fold increase in HER-2 expression and an approximately 231-fold increase in pHER-2 expression.

5. Growth inhibitory efficacy of natural phytochemicals

The data on the growth inhibitory efficacy of natural phytochemicals is summarized in Table II. Treatment with individual phytochemicals at their respective maximum

Table I. Hyper-proliferation in the HER-2-enriched 184-B5/HER model.

Quantitative end point	Cell line			
	184-B5[a]	184-B5/HER[a]	P-value	Relative to 184-B5
Population doubling (h)[b]	34.1±1.7	15.3±4.1	0.04	-55.1%
Saturation density (x10^5)[b]	23.7±1.3	38.6±1.7	0.02	+62.9%
G_1: $S+G_2/M$ (ratio)[c]	1.8±0.3	0.8±0.2	0.04	-55.6%
Sub-G_0 (%)[c]	18.9±2.6	0.7±0.2	0.01	-96.3%
Anchorage-independent Colony number (per 100 cells)[d]	Undetected	25.8±4.7		
HER-2 (log mean FU)[c]	0.3±0.1	53.8±2.5	0.005	+178X
p-HER-2 (log mean FU)[c]	0.2±0.1	46.5±2.2	0.005	+231X

[a]Mean ± SD, n=3 per treatment group. [b]Determined at day 7 post-seeding by the number of viable cells using trypan blue dye exclusion assay. [c]Determined at day 3 post-seeding by PI-positive or FITC-conjugated antibody positive cells using flow cytometry. [d]Determined at day 21 post-seeding by AI colony number using anchorage independent growth assay. Data were analyzed by a two-sample Student's t-test. AI, anchorage independent; HER-2, human epidermal growth factor receptor 2; p-HER-2, phosphorylated HER-2; FU, fluorescence units; PI, propidium iodide; FITC, fluorescence isothiocyanate; X, fold change. Data shown are summarized from a previous study (16).

Table II. Anti-proliferative effects of natural phytochemicals in 184-B5/HER cells.

Treatment	Concentration (μM)[a]	Biomarker					
		G_1: $S+G_2/M$[b]	P-value	δ EtOH	p-HER-2[c]	P-value	δEtOH
EtOH	0.1%	0.7±0.1		-	46.5±3.3		-
I3C	100	1.7±0.2	0.02	+1.4X	34.4±5.6	0.05	-26.0%
EGCG	20	1.5±0.2	0.02	+1.1X	18.7±0.9	0.02	-59.8%
GEN	10	2.9±0.4	0.01	+3.1X	6.9±0.3	0.01	-85.2%
CA	10	1.0±0.1		+42.8%	37.1±1.7	0.05	-20.2%
CSOL	10	1.0±0.1		+42.8%	36.0±1.6	0.05	-22.6%

[a]IC$_{90}$, maximum cytostatic. [b]Determined by FACS analysis of PI-positive cells. [c]Determined by immunofluorescence assay using FACS analysis of FITC-conjugated antibody positive cells. Mean ± SD, n=3 per treatment group. Data were analyzed by ANOVA and Dunnett's post-hoc multiple comparison test (α=0.05). FACS, fluorescence assisted cell sorting; PI, propidium iodide; FITC, fluorescence isothiocyanate; EtOH, ethanol (solvent control); p-HER-2, phosphorylated HER-2, I3C, indole-3-carbinol; EGCG, epigallocatechin gallate; GEN, genistein; CA, carnosic acid; CSOL, carnosol, X, fold.

cytostatic (IC$_{90}$) concentrations resulted in a substantial increase in the G_1:$S+G_2/M$ ratio ranging from approximately 43% for CA to approximately 3-fold for GEN. This increase in the G_1:$S+G_2/M$ ratio is indicative of cytostatic growth arrest. At the mechanistic level, phytochemical treatment resulted in the downregulated expression of p-HER-2, ranging from approximately 20% for CA to approximately 85% for GEN. In addition, these data facilitate the rank ordering of natural phytochemicals based on their efficacy for biomarker modulation. Thus, with regard to the G_1:$S+G_2/M$ ratio, the rank order is GEN>I3C>EGCG>CA=CSOL. For pHER-2 inhibition the rank order is GEN>EGCG>I3C>CSOL>CA. Thus, the rank order suggests distinct efficacy for p-HER-2 inhibition that may be responsible for the observed effect on cell cycle progression.

The data on the pro-apoptotic effects of the phytochemicals are summarized in Table III. Treatment with the phytochemicals results in a substantial increase of the cell population in the sub-G_0 (apoptotic) phase of the cell cycle ranging from approximately 8-fold for I3C to approximately 17-fold for GEN, relative to the solvent control. At the mechanistic level, the induction of cellular apoptosis was associated with a reciprocal modulation of apoptotic-specific proteins due to the reduced expression of the anti-apoptotic BCL-2 protein, ranging from approximately 23% for I3C to approximately 67% for GEN, and the increased expression of pro-apoptotic BAX protein, ranging from approximately 23% for I3C to approximately 76% for CSOL (Table IV). Thus, with regard to the induction of apoptosis, the rank order is GEN>CSOL>EGCG>CA>I3C. The rank order for the decrease in BCL-2 expression is GEN>CSOL>CA>EGCG>I3C, while the rank order for the increase in BAX expression is CSOL>GEN>CA>EGCG>I3C. Collectively, the rank order for the efficacy for induction of cellular apoptosis indicates distinct mechanisms of action for

Table III. Pro-apoptotic effects of natural phytochemicals in 184-B5/HER cells.

Treatment	Concentration (μM)[a]	Biomarker		
		Sub-G_0 (%)[b]	P-value	δEtOH
EtOH	0.1%	0.7±0.2		
I3C	100	6.4±1.8	0.02	+8.1X
EGCG	20	8.1±2.3	0.01	+10.6X
GEN	10	12.7±3.6	0.01	+17.1X
CA	10	7.2±2.0	0.02	+9.3X
CSOL	10	10.4±2.9	0.01	+13.8X

[a]IC_{90}, maximum cytostatic. [b]Determined at day 3 post-seeding by FACS analysis of PI-positive cells. Mean ± SD, n=3 per treatment group. Data analyzed by ANOVA and Dunnett's post-hoc multiple comparison test (α=0.05). FACS, fluorescence-assisted cell sorting, EtOH, ethanol (solvent control); I3C, indole-3-carbinol; EGCG, epigallocatechin gallate; GEN, genistein; CA, carnosic acid; CSOL, carnosol; BCL-2, B-cell lymphoma; BAX, BCL-2-associated X protein, X, fold change.

Table IV. Modulation of apoptosis associated protein by natural phytochemicals in 184-B5/HER cells.

Treatment	Concentration (μM)[a]	Biomarker					
		BCL-2[b]	P-value	δEtOH	BAX[b]	P-value	δEtOH
EtOH	0.1%	80.5±5.1		-	25.3±2.7		-
I3C	100	62.1±3.9	0.05	-22.8%	32.6±3.3	0.05	+22.8%
EGCG	20	51.0±3.2	0.05	-36.6%	35.2±3.6	0.04	+39.1%
GEN	10	26.7±1.7	0.01	-66.8%	44.1±4.5	0.01	+74.3%
CA	10	31.5±1.9	0.02	-60.9%	37.5±3.8	0.04	+48.2%
CSOL	10	29.9±1.9	0.02	-62.8%	44.5±4.5	0.01	+75.9%

[a]IC_{90}, maximum cytostatic. [b]Determined at day 3 post-seeding by FACS analysis of FITC-conjugated antibody-positive cells. Mean ± SD, n=3 per treatment group. Data are presented as log mean FU per 10^4 fluorescent events, and analyzed by ANOVA and Dunnett's post-hoc multiple comparison test (α=0.05). BCL-2, B-cell lymphoma; BAX, BCL-2 associated X protein; EtOH, ethanol (solvent control); I3C, indole-3-carbinol; EGCG, epigallocatechin gallate; GEN, genistein; CA, carnosic acid; CSOL, carnosol.

individual phytochemical. It is also notable that the growth inhibitory effects of these phytochemicals have been reported in the HER-2-negative human breast carcinoma derived cell lines, MCF-7, MCF-10 CA 1a and MDA-MB-231 (18-21). However, the effective concentrations differ from the ones presented for the 184-B5/HER model.

6. Drug-resistant stem cell model

In the preclinical models for HER-2-enriched breast cancer, the HER-2-specific antibody, trastuzumab, and the HER-2/EGFR dual-specific small molecule inhibitors display acquired drug resistance (22,23). Consistent with these data, the 184-B5/HER cells treated with the HER-2/EGFR small molecule inhibitor, lapatinib (LAP), provided the drug-resistant stem cell model. Long-term treatment with the maximum cytostatic (IC_{90}) concentration of LAP generated progressively growing LAP-R cells. The data summarized in Table V demonstrate an approximately 1-fold increase in the number of tumor spheroids, an approximately 3-fold increase in cluster of differentiation (CD44), a 3-fold increase in DNA binding homeobox nuclear

transcription factor (NANOG) and a 2-fold increase in octamer binding transcription factor-4 (OCT-4), relative to that observed in LAP-sensitive (LAP-S) cells. The cell surface marker, CD44, and the nuclear transcription factors, NANOG and OCT-4, represent well-established markers of the stem cell population (9). Thus collectively, these data indicate that the LAP-R cells may represent putative cancer stem-like cells in the present model.

7. Stem cell targeting testable alternatives

Frequent occurrence of acquired tumor resistance against pharmacological therapeutic agents (2,3,7,8) and the emergence of drug-resistant cancer stem cells (22,23) emphasizes an unmet need to identify stem cell targeting testable alternatives. Unlike pharmacological agents, natural phytochemicals, due to their low toxicity profiles, are less likely to induce acquired tumor resistance. Published studies on the rosemary terpenoids, CA and CSOL, have documented potent anti-proliferative and/or pro-apoptotic effects at cytostatic low μM concentrations on the present HER-2-enriched model (16). It is therefore of considerable relevance to examine the stem cell targeted

Table V. Drug-resistant stem cells derived from the lapatinib-resistant (LAP-R) phenotype.

		Expression of stem cell markers		
Phenotype	Tumor spheroids[a]	CD44[b]	NANOG[b]	OCT-4[b]
LAP-S	4.5±1.2	4.0±1.1	2.2±0.6	4.8±1.3
LAP-R	10.3±2.7	16.8±3.6	9.7±2.5	9.1±2.4
χ^2	7.88			
P-value	0.005	0.01	0.01	0.03
Relative to LAP-S	+1.3X	+3.2X	+3.4X	+1.8X

[a]Number of tumor spheroids at day 14 post-seeding. [b]Determined at day 3 post-seeding by flow cytometry of FITC-conjugated antibody-positive cells. Data are presented as log mean FU. Mean ± SD, n=3 per treatment group. Tumor spheroids were analyzed by the χ^2 test. CD44, NANOG andOCT-4 were analyzed by a two-sample Student's t-test. CD44, cluster of differentiation 44; NANOG, DNA binding homeobox transcription factor; OCT-4, octamer binding protein-4; LAP-S, lapatinib-sensitive; LAP-R, lapatinib-resistant; FU, fluorescent units; X, fold. Data shown are summarized from a previous study (12).

Table VI. Modulation of stem cell markers in the lapatinib-resistant (LAP-R) phenotype.

		Expression of stem cell markers		
Treatment	Concentration	Tumor spheroids[a]	CD44[b]	NANOG[b]	OCT-4[b]
DMSO	0.1%	14.8±1.9	20.8±4.4	11.8±3.1	14.2±3.8
CSOL	5 μM	1.9±0.2	2.9±0.6	2.7±0.7	2.9±0.9
χ^2		7.74			
P-value		0.010	0.010	0.020	0.020
Relative to DMSO		-87.2%	-86.0%	-77.1%	-79.6%

[a]Number of tumor spheroids determined at day 14 post-seeding. [b]Determined at day 3 post-seeding by immuno-fluorescence assay of FITC-conjugated antibody-positive cells. Data are presented as log mean FU. Mean ± SD, n=3 per treatment group. Tumor spheroids were analyzed by the χ^2 test. CD44, NANOG and OCT-4 were analyzed by a two-sample Student's t-test. CD44, cluster of differentiation 44; NANOG, DNA binding homeobox transcription factor; OCT-4, octamer binding protein-4; DMSO, dimethyl sulfoxide (solvent control); CSOL, carnosol. Data shown are summarized from a previous study (12).

efficacy of natural phytochemicals. The data presented in Table VI demonstrate that in response to a treatment with CSOL the LAP-R cells exhibit an approximately 87% decrease in the number of tumor spheroids, an 86% decrease in CD44 expression, a 77% decrease in NANOG and an 80% decrease in OCT-4 expression, relative to the solvent-treated controls. Collectively, these data provide a proof of concept for a mechanistic lead for the rosemary terpenoid, CSOL, as a stem cell targeting natural alternative. With regard to the cancer stem cell-targeted efficacy of natural products, it is noteworthy that several mechanistically distinct natural products have documented efficacy against drug resistant stem cell population. For example, the vitamin A derivative, all-trans retinoic acid (ATRA), inhibits gastric cancer stem cell growth via inhibiting CD44, aldehyde dehydrogenase-1 (ALDH1), Kruppel-like factor-4 (KLF-4) and sex determining region Y-box-2 (SOX-2) expression (24). Sulphoraphane present in broccoli inhibits tumor spheroid formation and ALDH-1 expression in pancreatic and prostate cancer stem cells (25), inhibits NANOG, ALDH-1, Wnt-3 and Notch expression in triple-negative breast cancer stem cells (26), and benzyl isothiocyanate present in

cruciferous vegetables inhibits breast cancer stem cells via upregulation of KLF-4-p21 axis (27). The inhibition of established stem cell markers provides potential mechanistic leads for the stem cell-targeted efficacy of natural products.

In addition to natural phytochemicals, nutritional herbs may represent testable alternatives for therapy resistant breast cancer. Herbal aqueous extracts are extensively used in traditional Chinese medicine for a variety of health issues in women, including cancer (28). Non-fractionated aqueous extracts from Chinese nutritional herbs, simulating patient consumption, exhibit anti-proliferative and pro-apoptotic effects in cellular models for molecular subtypes for clinical breast cancer. In the MCF-7 model for the luminal A subtype, *Epimedium grandiflorum* extract inhibits cancer growth via altering estrogen metabolism and generating anti-proliferative metabolite 2-hydroxyestrone (29). In the MCF-7[AROM] model for aromatase-expressing post-menopausal breast cancer, Taheebo-NFD-Marugoto (TNM), an extract from the *Tabebuia avellanedae* tree, exhibits anti-proliferative effects via inhibition of estrogen regulated genes and pro-apoptotic effects via increase in pro-apoptotic caspase 3/7 activity and

upregulation of BAX gene. Additionally, TNM exerts superior inhibitory effects on aromatase activity than clinical aromatase inhibitors (30). In the MDA-MB-231 model for triple-negative breast cancer, *Cornus officinalis* extract induces anti-proliferative and pro-apoptotic effects via the inhibition of cyclin D1 and pRB, and via the upregulation of caspase-3/7 activity and BAX expression, respectively (31). Collectively, these data provide mechanistic leads for the efficacy of nutritional herbs on models for breast cancer subtypes, and thereby, provide a rationale for investigations on developed stem cell models. It is also notable that the stem cell-specific transcription factors, OCT-4, SOX-2 and NANOG, represent markers for poor survival in HER-2-positive clinical breast cancer (32).

8. Conclusions and future prospects

The data reviewed herein lead to the conclusion that the cellular model for the HER-2-enriched breast cancer subtype in general and drug-resistant stem cell model in particular, provide valuable experimental approaches for identifying natural phytochemicals as testable alternatives for treatment of chemotherapy therapy resistant breast cancer.

Human tissue-derived cellular models represent valuable experimental approaches to reduce preclinical data extrapolation for their clinical translatability. However, to further enhance clinical translatability, future investigations utilizing patient derived *ex vivo* breast cancer organoid models (33,34) are likely to provide valuable clinically translatable data on stem cell-targeting natural products.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the author on reasonable request.

Author's contribution

The author NT contributed towards study conception, experimental design and prepared the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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