# The Implications of Hyponitroxia in Cancer

**Bryan Oronsky\*, Gary R. Fanger\*, Neil Oronsky†, Susan Knox‡ and Jan Scicinski\***

\*RadioRx, Inc., Mountain View, CA, USA; †Department of Radiation Oncology, Stanford University Medical Center, Stanford, CA, USA; ‡CFLS, San Jose, CA, USA

## Abstract

Tumors are spatially heterogeneous, with regions of relative hypoxia and normoxia. The tumor microenvironment is an important determinant of both tumor growth and response to a variety of cytotoxic and targeted therapies. In the tumor microenvironment, reactive oxygen species and nitric oxide (NO) are important mediators of the level of expression of many transcription factors and signaling cascades that affect tumor growth and responses to therapy. The primary objective of this review is to explore and discuss the seemingly dichotomous actions of NO in cancer biology as both a tumor promoter and suppressor with an emphasis on understanding the role of persistently low NO concentrations or hyponitroxia as a key mediator in tumor progression. This review will also discuss the potential role of hyponitroxia as a novel therapeutic target to treat cancer and outline an approach that provides new opportunities for pharmacological intervention.

*Translational Oncology (2014) 7, 167–173*

## Introduction

This review highlights the function of hyponitroxia as a proneoplastic effector, summarizes therapeutic strategies to increase intratumoral nitric oxide (NO) to mitigate, at least in part, the effect of hyponitroxia on angiogenesis and malignant progression, and makes the case for hyponitroxia a high-priority target in cancer therapy that may be as, if not more, important than hypoxia.

As in tumors, NO also plays an important role in normal tissues. Under physiological conditions, low levels of NO are produced from L-arginine by constitutively expressed NO synthase in neuronal cells (nNOS, also known as NOS1) and endothelial cells (eNOS or NOS3) [1], which contribute to the regulation of normal physiological processes through cell signaling (Figure 1). Higher levels of NO are produced by an inducible nitric oxide synthase (iNOS or NOS2) [1]. NO can stimulate pathways resulting in either cell growth or cell death, depending on the relative level of NO and a variety of associated factors [2].

## The Hyponitroxia and Hypoxia Axis

In tumors, hyponitroxia is relative rather than absolute: low levels of NO (<100 nM) [3] are produced by three NOS enzymes described above [4]) and associated with the oxidative burst of macrophages. At the low concentrations of NO found in tumors, NO mediates redox signaling pathways linked to the proangiogenic activities of vascular endothelial growth factor and inhibition of thrombospondin 1 [5], promoting malignant conversion, tumor progression [6], and

resistance to therapy in multiple cancers including prostate [7], colonic, lung [8], and mammary adenocarcinomas [8,9]. Other candidate oncogenic functions of NO include cell proliferation, invasion and metastasis, and stem cell renewal [3]. Hyponitroxia thus represents a modified form of hormesis [10], a dose-response model characterized by a beneficial effect at low doses and a detrimental effect at high doses. NO also exerts a direct effect on responses to hypoxia through changes in expression of hypoxia inducible factor, alpha subunit (HIF-1α). Mimicking and attenuating hypoxia [11], NO drives HIF-1α signaling, by inhibition of prolyl hydroxylase 2 [12], resulting in a more aggressive and resistant phenotype (Figure 2).

Hypoxia catalyzes the oncogenicity of NO: in addition to L-arginine, molecular oxygen is an essential substrate for the activity of NOSs, and exposure to low-oxygen tension limits endogenous NO production by these enzymes [13,14]. However, in the absence of complete anoxia, a rare state even in tumors, NO synthesis is only inhibited rather than abrogated [14], resulting in the constitutive induction of the enzyme guanyl cyclase (GC) [15] and the accumulation of its downstream mitogenic effector

cyclic guanosine monophosphate. *S*-nitrosylation of caspases, leading to their inactivation, has also been proposed as a mechanism by which NO can block apoptosis and result in tumorigenesis [16]. In addition, hypoxia also redirects macrophage L-arginine metabolism from NOS to arginase [17], an enzyme that converts L-arginine to urea, leading to decreased arginine availability as a substrate for NO production.

Thus, as an inactivating mechanism for endogenous NO production, hypoxia acts as a protumorigenic stimulus, potentiating the destructive potential of NO [18], separate from its effects on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [19] and HIF-dependent transcriptional pathways.

However, the reverse is true as well: hyponitroxia exacerbates hypoxia through alterations in blood flow and oxygen consumption through NO mitochondria-mediated pathways [20,21]. Therefore, hypoxia and hyponitroxia are closely related and can affect a variety of downstream targets—either simultaneously or sequentially.

### NO-Mediated Effects

In the past two decades or so, NO has been implicated in a wide array of potential effects on cancer suppression and progression. It is now generally agreed that NO has a highly context-dependent dose–response stimulation-inhibition relationship with cytotoxicity at high doses and mitogenicity at low doses [22]. Thus, NO has the ability to both promote and suppress cancer.

However, these binary either/or descriptions are an oversimplification. At low constitutive levels induced by hypoxia in tumors, NO levels are optimal for the mediation of aberrant, proliferative signaling. In contrast, levels either above or below this optimal range can have the opposite effect and activate signal transduction pathways that contribute to/result in growth inhibition or cell death.

### The Threshold Dose and Cytotoxicity

NO is a radical with a free electron capable of interacting with reactive oxygen species (ROS) such as the superoxide anion to form a variety of highly reactive nitrogen oxides (NOx). The term *nitrosative stress* refers to the formation of NOx compounds such as peroxynitrite (ONOO⁻), nitrogen dioxide ($NO_2$), and dinitrogen trioxide ($N_2O_3$) that are responsible for cytotoxic nitration and oxidation reactions [23] leading to apoptosis and cell death. In particular, the formation of peroxynitrite is a first-order reaction [23] dependent on the concentrations of NO and the superoxide anion and, therefore,

on oxygen tension, because in the presence of hypoxia, both NO and ROS such as the superoxide anion will be less prevalent.

Xie et al [24] demonstrated that transfection of murine K-1735 melanoma cells with inducible NOS leading to the generation of high levels of NO resulted in suppression of tumorigenicity and metastasis. The cytotoxicity of higher concentrations of NO is consistent with the assumption that the toxic effect becomes apparent above a threshold dose of NOx. This balance between mitogenic and toxic effects of NO in tumor cells is potentially attributable to an increased susceptibility to free radical damage due to severe impairment of the antioxidant defense system [25] compared with healthy cells.

In cancer cells, reactive oxygen/nitrogen species "reprogram" the cellular metabolism toward a dependence on glucose use, termed the *Warburg effect*, a signature of virtually all tumors and the basis of fluorodeoxyglucose positron emission tomography imaging, to support anabolic proliferation. The fact that this core feature of tumors, metabolic reprogramming, is dependent on redox signaling implies that ROS/reactive nitrogen species (RNS) levels are higher in tumors than in healthy tissue, resulting in a differential sensitivity to oxidant stress [26]. Indeed, the presence of high levels of ROS in tumors has been linked with cell cycle arrest and apoptosis [27].

However, NOx cytotoxicity may not require superelevated doses but rather approximate "normalization" to physiological levels [27], because shifts in a particular direction can have important consequences. For example, Frederiksen et al. demonstrated that NO enrichment through low concentrations of the NO mimetics glyceryl trinitrate (GTN) and isosorbide dinitrate attenuated hypoxia-induced resistance to doxorubicin in prostate cancer mouse models.

At the other end of the spectrum, Kashiwagi and Jain [28] described radiosensitization in glioma xenografts through the normalizing effects of NOS inhibition on the tumor vasculature. The cytotoxicity of NO below a certain threshold is consistent with the assumption that lower concentrations of NO reduce signal transduction below a physiological baseline, leading to a loss of the aberrant induction of proangiogenic [5] signaling [29] networks that promote malignant progression (Figure 3).

This emerging background of conflicting preclinical evidence that both anti-NO–centered and pro-NO–centered therapeutic strategies are therapeutically effective has resulted in the initiation of human clinical trials with both NO donors and NO inhibitors such as
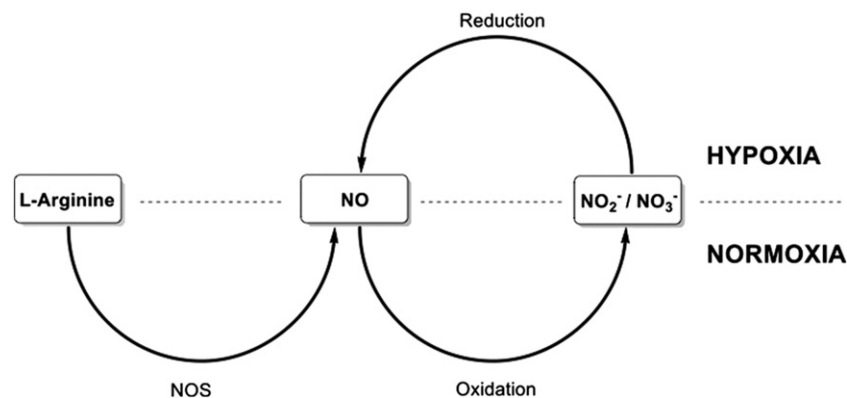


**Figure 1.** The nitrate-nitrite-NO pathway. NO is generated from the precursor L-arginine by the enzyme NOS under normoxic conditions. Under these conditions, NO is oxidized to nitrite and nitrate. Under hypoxia, nitrite is reduced by a variety of NOS-independent processes to form NO [2].

**Figure 2.** The relationship between nitric oxide and hypoxia.

nitroglycerin (NTG), N-nitro-L-arginine (L-NNA), and RRx-001 to push the tumor out of its "hormetic comfort zone."

### NO and Epigenetics

As an operational definition, epigenetics comprises heritable alterations in gene expression not due to changes in the underlying DNA sequence. These epigenetic alterations may involve changes in DNA methylation patterns, altered mRNA expression, and modifications of the histones around which the DNA is wrapped. NO has been shown to be an epigenetic factor on the basis of its ability to influence DNA methylation, microRNA and histone modification in normal [30] as well as tumor tissues [31], acting directly [32] or through induction of NOSs [33]. As a consequence of these mechanisms, therapies that result in global epigenetic changes in the tumor microenvironment or ecosystem [34] due to selective delivery or inhibition of NO may alter the tumor phenotype in such a way that it becomes sensitized or resensitized to subsequent chemotherapy, leading to improved overall survival [31,32,35]. Furthermore, it is possible that some epigenetic effects (e.g., DNA methylation, histone modifications, and micro-RNAs), might have immunomodulatory effects and could potentially affect immune cell and cytokine function in the tumor microenvironment in such a way as to facilitate antitumor immune responses.

### NO and p53

In response to DNA damage, the p53 tumor suppressor protein activates checkpoint-mediated $G_1/S$ arrest or apoptosis to prevent proliferation of cells with a damaged genome. p53 transcriptionally activates downstream genes such as *p21*, which bind to and inhibit several cyclin dependent kinase complexes. p53 is also implicated in the induction of cellular senescence, also through *p21* gene activation. An increase in NO levels may lead to tumor senescence, characterized by p53 activation, through p53 nuclear retention [35] and the secretion of proinflammatory cytokines such as Interleukin 6 (IL-6) and IL-8, which stimulate the immune system.

The modulation of senescence through selective NO delivery to tumors may improve cancer outcomes through a reduction in toxicity-related side effects and stimulation of immune activity, limiting the growth of tumors that have bypassed many major tumor suppressor blocks [36–38].

### NO and the Immune Response

A large number of studies have implicated NO as having an important role in immune function [39]. As initially described, macrophages were shown to produce NO in response to infection, which functions directly to kill or suppress replication of infectious pathogens. It was subsequently determined that other immune cells including neutrophils, eosinophils, nonhematopoietic cells, and even certain subsets of dendritic cells express NO, further supporting the notion that NO may have important modulatory actions on the immune system. The role of NO in the immune system is complex, and effects of NO on immune function can be enhancing or suppressing, depending on the level of exposure and the context in which it is available. For example, studies have shown that NO suppresses
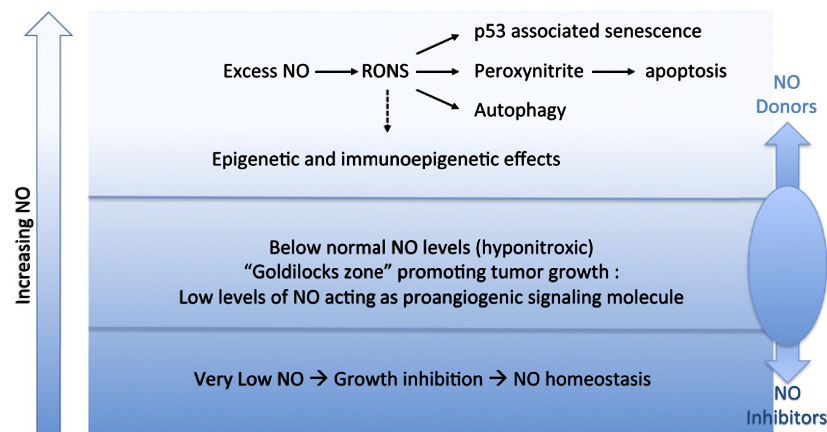


**Figure 3.** Hyponitroxia and the tumor response: How low can you go? Physiologic or very low concentrations of intratumoral NO inhibit tumor growth, whereas subphysiologic but detectable levels promote tumor growth through induction of angiogenesis and action on signaling cascades.

transforming growth factor β–mediated induction of transcription factor forkhead box P3 (Foxp3+) regulatory T cell (Treg) and drives differentiation toward the T helper cells 1 (Th1) lineage. In addition, in the presence of NO, transforming growth factor β–driven Th17 differentiation can predominate over Th1 as NO competes with IL-6 to refine the direction of differentiation [40]. Thus, there is important relevance in understanding the immunologic role that NO may play as a potential therapeutic target for the treatment of inflammatory disease or in the context of cancer with respect to the tumor microenvironment.

Mechanisms by which NO can impact immune function include changes in signaling pathways and transcription factors that, understandably so, can be similar to those that mediate antigen-dependent differentiation of T cells. NO can effect modulation of signaling cascades like mitogen-activated protein kinase, phosphoinositide 3-kinase, and janus kinase/signal transducer and activator of transcription pathways [41]. In addition to regulating p53 activity described above, NO can mediate a variety of control mechanisms on NF-κB including inhibition of DNA binding of NK-κB through S-nitrosylation of the p50 subunit, activating p21 Ras, and controlling inhibitor of kappa B (I-kB) or I-kB kinase [42,43]. The expression of such key molecules that control the fate of immune cells including B-cell lymphoma 2, B-cell lymphoma-extra large, and BCL2-associated X protein can also be impacted by exposure to NO [44].

As above, epigenetic effects may have modulatory effects on the immune system. Several lines of evidence support this concept: T and B cell differentiation are influenced by epigenetic mechanisms as well as the transcriptional control of *Foxp3* gene expression [45,46], which plays a key role in CD4 + T cell differentiation into Treg cells [47]. Thus, these events can have broad impact on the survival and activity of T cells, as well as other immune cells.

Another immune-related mechanism relevant for discussion can be found in the context of cancer where elevated NO levels have been shown to have antitumor activity. NO can sensitize tumor cells to immune-mediated killing through Fas-, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, and TNF-α–dependent mechanisms. The mechanism by which NO increases Fas sensitivity is due to inhibition of NF-κB and Yin Yang 1 that allows for increased levels of death-inducing Fas on the surface of tumor cells [48]. Reduction of the transcriptional repressor Yin Yang 1 also allows for increased expression of Trail on tumors and hence enhanced sensitivity to Trail-mediated apoptosis [49]. Because many tumors have mechanisms to circumvent apoptosis, elevated levels of NO could theoretically resensitize tumors to the induction of apoptosis.

## Nitroglycerin

NTG, or GTN, is an approved antianginal NO-donating nitrate ester [50] repurposed for evaluation as a single agent and chemosensitizer in late-stage cancer clinical trials.

### Monotherapy

In a phase II study, patients with prostate cancer who had failed primary therapy were treated with a low dose of sustained delivery GTN resulting in a significant decrease in prostate-specific antigen. The authors suggested that, although low-dose NO had no direct cytotoxic effect, NO decreased the emergence of a more malignant phenotype, including invasion and metastases [2], potentially by "normalizing" or "boosting" NO to physiological ranges. An

alternative hypothesis supporting these observations is that prolonged and sustained delivery of NO paradoxically resulted in inhibition of NO signaling through tachyphylaxis due to feedback inhibition of GC [2]. The latter possibility suggests itself as a consequence of the observations of Sonveaux et al., who have demonstrated that ionizing radiation activates proangiogenic signaling cascades through up-regulation of NOS in endothelial cells and NO production in the tumor vascular bed [51]. These studies suggest that it may be necessary to exceed a minimum threshold dose of NO before a switchlike response from a tumor stimulant to cytotoxicant is elicited.

### Chemosensitization

The effect of NO supplementation on the efficacy of chemotherapy was studied in a double-blind phase II randomized study of 120 patients with stage IIIB/IV non small cell lung cancer (NSCLC) [52], randomly assigned to a hybrid regimen of alternating courses of vinorelbine and cisplatin with either an NTG patch or placebo. Both time to disease progression and overall response rate were found to be significantly increased in the NTG arm. This marked effect of NO could be attributed to a normalization of NO levels from low to a normal physiological range in the tumor or, alternatively, an effect on GC and cyclic guanosine monophosphate production through feedback inhibition. Both scenarios would lead to disruption of the proangiogenic redox signaling circuitry. Although the investigators on the study mention a phase III trial, an extended search in PubMed and clinical trial listings found two recruiting phase II studies in non–small cell lung cancer and prostate cancer.

## N-Nitro-ʟ-Arginine

The reliance of tumors [53] on NO-mediated mechanisms of progression and metastasis prompted an evaluation of ʟ-NNA, a competitive inhibitor of NOS with selectivity for the neuronal and endothelial isoforms of the enzyme, in a phase 1 study of patients with NSCLC. Serial assessment with dynamic contrast-enhanced computed tomography demonstrated decreased vascular blood volume by 40%, an effect that was sustained 24 hours posttreatment [54]. It is not known whether this decrease in blood volume was associated with tumor shrinkage.

Extrapolation from these data suggests that tumors can only thrive within a hyponitroxic "comfort zone" of signaling cell strength; attenuation below and elevation above this level result in cell death or senescence [55]. Inhibition of NO synthesis has catastrophic effects on the tumor vasculature, which can be attributed to the involvement of NO in tumor angiogenesis and the maintenance of vasodilator tone of tumor blood vessels.

The sustained disruption of the tumor vasculature was preceded by a mild transient increase in systemic blood pressure; this discrepancy was attributed to a differential dependence on NO in healthy and cancerous tissues [56]. Unlike the cardiovascular system, which is subjected to tightly regulated homeostatic controls [56], the patency of vessels within tumors is largely regulated by increased expression of NO. Therefore, the consequence of NO inhibition was a conversion of net vasodilation to vasoconstriction, with a collapse of tumor blood flow.

## RRx-001

RRx-001 [57] is an aerospace industry–derived small-molecule redox regulator with NO-donating properties that has recently completed a phase I clinical trial in patients with a variety of solid tumors. In

addition to generating ROS, RRx-001 has a novel mechanism of action that involves selective and specific modification of hemoglobin in a subpopulation of RBCs, resulting in a catalytic, hypoxia-driven overproduction of NO [58]. This, in turn, leads to excess NOx, free radicals (RNS), diffusible metabolites, chemokines, and cytokines, all of which are preferentially toxic and selectively target the tumor microenvironment in a manner that mimics, with NOx instead of oxygen, the "respiratory burst" associated with intracellular killing of bacteria by phagocytes. The basis for therapeutic selectivity is controlled release of these endothelial cytotoxins under conditions of hypoxia and free radical overload—stress conditions that are unique to the aberrant tumor microvasculature.

RRx-001 acts as an NO donor that irreversibly binds to and allosterically modifies its target, the β $Cys^{93}$ residue on deoxygenated hemoglobin [59]. Although deoxygenated hemoglobin can function as a nitrite reductase converting the inorganic anion nitrite into NO under hypoxic conditions, the binding of RRx-001 to this residue greatly amplifies and accelerates this catalytic reaction [58]. This *in situ* generation of ROS/RNS under hypoxia shifts the biocharacter of the tumor microenvironment from habitable to inhabitable, whereas the ultrashort lifetime of ROS and RNS confines their activity to the tumor, sparing normal tissues from toxicity.

Therefore, RRx-001 can amplify oxidative and nitrosative stress under low-oxygen conditions that are specific to the tumor microenvironment. In addition, RRx-001 selectively depletes the antioxidant glutathione (reduced glutathione), resulting in a systemic increase of ROS [59] that can also exert an antitumor effect through the exquisite sensitivity of tumors to perturbations in oxidative stress [55,57].

Preliminary data suggest that RRx-001 acts in a stress-response pathway, presumably through NO release, that promotes activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 and the tumor suppressors p53 and p21, supporting the emerging idea that RRx-001 leads to the onset of replicative senescence, resulting in cell cycle arrest or apoptosis in addition to other mechanisms of cell death. In a phase I trial, many patients had stable disease, with the median overall survival of 16.8 months, suggesting a possible survival advantage (RadioRx, 2013). In addition, three patients subsequently responded to chemotherapy regimens to which they had previously failed, suggesting that the prior RRx-001 treatment had resulted in resensitization. We have hypothesized that RRx-001 induced high tumor levels of NO/RNS that resulted in epigenetic changes in the patients' tumors that made them more sensitive to subsequent therapies. This is an active area of ongoing investigation.

## Conclusions and Future Directions
NO has only recently been recognized as a potentially useful target for treating cancer. A recent search of clinical trials listed on ClinicalTrials.gov revealed more than a hundred studies involving cancer and hypoxia. By contrast, there are less than 10 involving cancer and NO.

Rather than characterizing hyponitroxia as an accomplice to hypoxia, it might be more appropriate to describe the relationship of ROS and NO in terms of codependency because they interact cooperatively and reciprocally to mutually modulate biologic effects. Like an endocrine feedback system, the ROS/RNS axis operates through dose-responsive facilitative and inhibitory interactions. For example, NOS is inhibited under hypoxia and stimulated under oxic

conditions, whereas NO interferes with mitochondrial respiration and increases oxygen availability. In addition, NO and superoxide anion scavenge each other [60]. In this tightly coupled control, modulation of one element of the axis should induce a concomitant change of the other in the same direction.

It is important to point out that tumors are spatially heterogeneous with areas of hypoxia and normoxia, which can be stable or transient. This heterogeneity, with associated regions of hypoxia, is an important contributor to resistance of many tumors to therapy. It is anticipated that NO/RNS levels are also heterogeneous in tumors. It will be important to study the effect of NO/NRS-generating agents on this heterogeneity, which may be particularly relevant to understanding how modulation of NO levels within tumors may affect tumor responses when these agents are given concurrently or sequentially with other therapies.

In the literature, the response to NO has been described as biphasic [61], with homeostasis at low doses and toxicity at higher doses. In terms of tumors, NO responses may more closely follow a triphasic response, with cytotoxicity at physiological (and higher) doses, maintenance of homeostasis at hyponitroxic doses, and cytotoxicity again at even lower doses. The exploitation and modulation of hyponitroxia are potentially promising and exciting anticancer strategies, especially because direct approaches to improve the oxygenation of tumors with hyperbaric oxygen or a variety of methods of enhanced delivery have by and large been unsuccessful [62]. By contrast, hyponitroxia may be a more accessible target than hypoxia, indirectly resulting in an alteration of the oxygen status of the tumor.

Because the steady-state concentration of NOx conducive to invasion, angiogenesis, and metastasis is confined to a narrow hyponitroxic range, any significant perturbation in the fully coupled ROS/RNS axis in either direction, below or above, is likely to result in antitumor responses, especially in combination with chemotherapy or radiation therapy as mentioned above.

In summary, there is a need for discovery identification and study of new agents that target hyponitroxia and exert their anticancer activity through modulation of intratumoral NO, thereby tipping the balance from tumor cell survival to cell death and senescence. In addition, further research into new imaging modalities that can capture the effects of NO on tumors will be required [63]. Research into the use of NO/RNS modulation for purposes of signal amplification and attenuation with GTN (and other organic nitrates), RRx-001, and L-NNA may help to elucidate the molecular mechanism of action of these agents to enable optimization of their use both as single agents and in combination with other therapies on the basis of a better understanding of the underlying biology of hyponitroxia and facilitate the clinical development of new treatment options on the basis of this innovative approach.

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