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Exploiting the cancer niche: tumor-associated macrophages and hypoxia as promising synergistic targets for nano-based therapy

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Abstract The tumor microenvironment has been widely exploited as an active participant in tumor progression. Extensive reports have defined the dual role of tumor-associated macrophages (TAMs) in tumor development. The protumoral effect exerted by the M2 phenotype has been correlated with a negative outcome in most solid tumors. The high infiltration of immune cells in the hypoxic cores of advanced solid tumors leads to a chain reaction of stimuli that enhances the expression of protumoral genes, thrives tumor malignancy, and leads to the emergence of drug resistance. Many studies have shown therapeutic targeting systems, solely to TAMs or tumor hypoxia, however, novel therapeutics that target both features are still warranted. In the present review, we discuss the role of hypoxia in tumor development and the clinical outcome of hypoxia-targeted therapeutics, such as hypoxia-inducible factor (HIF-1) inhibitors and hypoxia-activated prodrugs. Furthermore, we review the state-of-the-art of macrophage-based cancer therapy. We thoroughly discuss the development of novel therapeutics that simultaneously target TAMs and tumor hypoxia. Nano-based systems have been highlighted as interesting strategies for dual modality treatments, with somewhat improved tissue extravasation. Such approach could be seen as a promising strategy to overcome drug resistance and enhance the efficacy of chemotherapy in advanced solid and metastatic tumors, especially when exploiting cell-based nanotherapies. Finally, we provide an in-depth opinion on the importance of exploiting the tumor microenvironment in cancer therapy, and how this could be translated to clinical practice.

Keywords:

Cancer hypoxia · Tumor-associated macrophages · Tumor microenvironment · Targeted cancer therapy · Nanomedicine

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1. Introduction

Cancer is amongst the leading causes of mortality and morbidity worldwide. The latest statistics point to an estimated 14.1 million new cases and 8.2 million cancer-related deaths [1]. It has been characterized by an uncontrolled cell proliferation, which is often associated with vascular abnormalities and cell invasiveness. In the past few years, the hall marks of cancer have been revised and an active participation has been assigned to the tumor microenvironment (Fig. 1) [2]. Studies have shown that cancer cells evolved to promote angiogenesis, metastasis and survival in response to several factors within the tumor microenvironment, such as pH, growth factors, oxygen levels and the presence of immune cells [3-5]. Tumor cells adaptation to the environment is considered essential to maintain their survival and growth. For instance, tumor cells grow under low levels of oxygen and nutrients, develop new blood vessels, a process called 'de novo angiogenesis' to compensate for the lack of oxygen and nutrients. These newly formed blood vessels contain discontinuous endothelium, which renders them leaky in nature. This vascular hyperpermeability, in combination with impaired lymphatic drainage, are known as enhanced permeation and retention effect (EPR) [6]. Surgery, radiotherapy, and classical chemotherapy are still the first options for many types of tumors [7]. However, the associated side effects and the emergence of multidrug resistance (MDR), have limited the clinical use of most common therapeutic compounds [8]. Therefore, there is an unmet need to develop novel approaches and therapeutics to target the tumor cell and its microenvironment. The heterogeneity and complexity of tumor microenvironment promote cancer survival and progression. Lately, research has focused on the involvement of tumor-associated macrophages (TAMs) in cancer progression. Studies have explored the link between the secretion of TAM chemoattractants by the tumor cells and the consequent upregulation of tumor-promoting genes by these immune cells, in response to the microenvironment stimuli [9, 10]. Additionally, hypoxia has been found as a critical factor for the survival of large tumor masses and therefore a key target for the development of targeted therapies [11]. Furthermore, high infiltration of TAMs are found in hypoxic tumor cores, promoting resistance to classical chemotherapeutics. This microenvironment may be used to develop sophisticated fine-tuned nano-based systems, capable of enhancing therapeutic extravasation into tumor hypoxic cores. Great efforts should be made to promote the rational design of delivery systems that could achieve high therapeutic efficacy by simultaneously targeting TAMs and hypoxia.

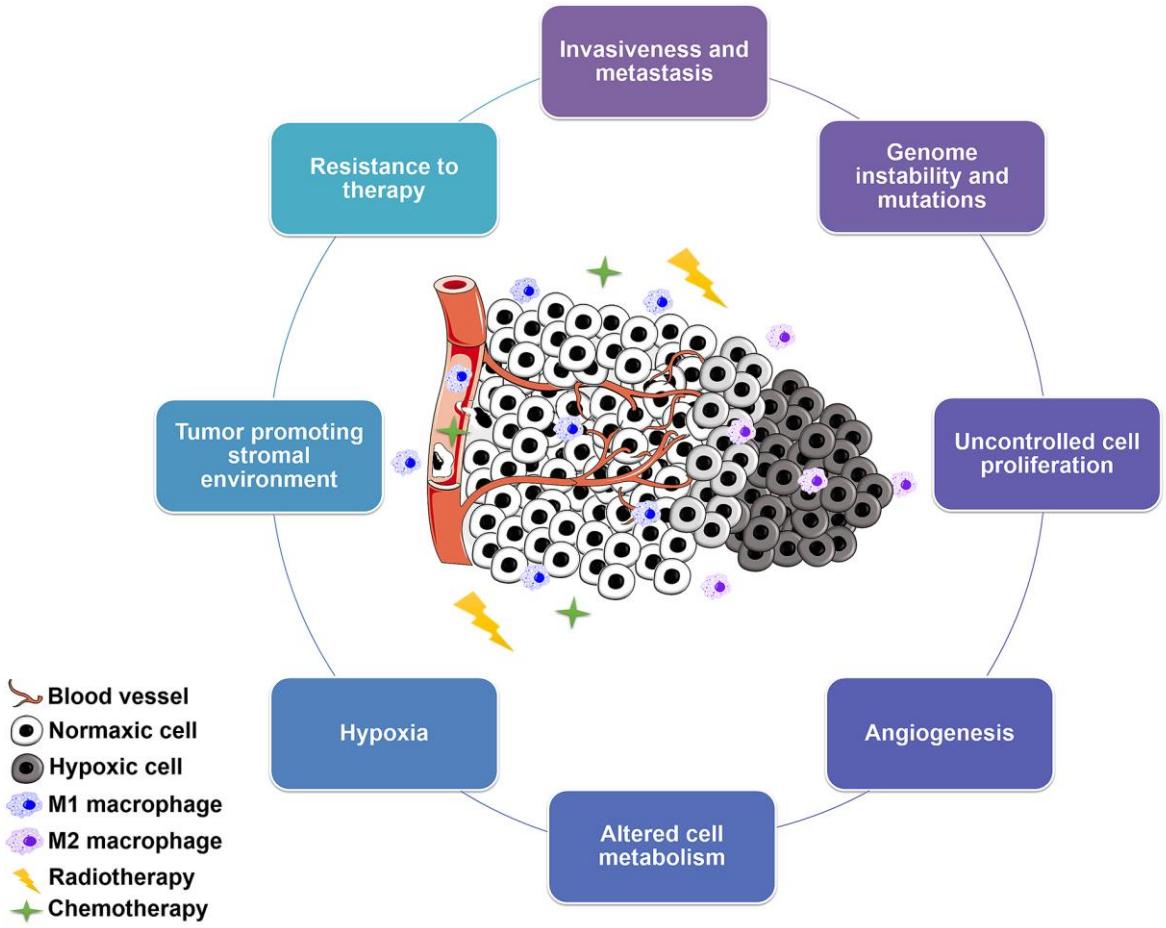

Fig 1. Main hallmarks of cancer. The cancer niche is a complex network of endothelial, stromal and malignant cells, comprised of evolutionary genomic features that enhance survival and thrive tumor cells to uncontrolled proliferation and metastasis. This enriched tumor microenvironment supports a shifted metabolism in cells, which allows a quick preadaptation and survival under nutrient and oxygen deprivation (hypoxia) which lead to therapeutic resistance.

2. Cancer Hypoxia

Structural abnormalities in tumor vessels lead to reduced oxygen diffusion to tumor cells and eventually, necrotic cores. It has been fifty some years since Thomlinson and Gray first postulated the role of hypoxia in human tumors [12]. Hypoxia has an active role in oncogenesis and contributes to the overall survival of tumors. In normal tissues, oxygen levels are heterogeneous and physiological pO2 can range between 20 mmHg in the liver and brain to 70 mmHg in the kidney (3.1-8.7 % O2). In contrast, a decrease to about 10-30 mmHg of pO2 is observed in tumors. Most importantly, 82% of all oxygen readings taken from solid tumors, present a 0.33 % O2 reading (as low as 2.5 mmHg) [13, 14]. The level of hypoxia within tumors increases during tumor progression. Chronic hypoxic cells have been described as prone to higher proliferation and survival. Aggressive phenotype with increased resistance to therapy has been associated with patients with highly hypoxic tumors, highlighting the clinical significance of hypoxia [15].

Hypoxia-inducible factors (HIFs), essentially HIF-1 have been linked to hypoxic tumor microenvironments. It is commonly overexpressed in solid and metastatic tumors including breast, prostate, colon, lung, pancreatic, head and neck cancer [16]. This molecule functions as a heterodimeric transcription factor composed of HIF-1α and HIF-1β, whose dimerization is regulated by an oxygen-dependent prolyl hydroxylase. When oxygen levels decrease, HIF-1α accumulates and translocates to the nucleus, where it forms the active transcription factor HIF-1 by binding to HIF-1β. This molecule regulates a plethora of genes in cancer biology and metabolism, controlling the proliferation rate, metastasis, and aggressiveness of cancer cells [17]. It also potentiates tumor cells resistance to radio and chemotherapy [18-20]. Although some controversy exists on using HIF-1 as an actual target for hypoxia, many novel therapies have been exploited, which will be discussed below, suggesting the relevance of hypoxia in cancer therapy.

Several therapeutic agents, such as anti-angiogenesis (VEGF inhibitors) [14, 21, 22], tumor MAC depleting agents [23], and androgen deprivation therapy [19], have shown to induce tumor hypoxia. Most of these drugs work by shutting the blood supply to the tumor, reducing nutrient and oxygen delivery to the cancer cells, which results in tissue hypoxia. Photosensitizers are another class of cancer therapeutics, which consumes tumor oxygen upon light activation to produce reactive oxygen species (ROS), leaving lower oxygen levels in the tumor microenvironment [24, 25]. Drug-induced hypoxia has been considered indeed as a key factor that reduces the therapeutic efficacy of a wide range of anti-cancer treatments. However, recent studies have shown that careful selection of the combinatory treatment, as well as, their sequence, could convert hypoxia from a challenge into a potential therapeutic target. For instance, a recent study combining Topotecan (hypoxia-induced factor inhibitor) with Bevacizumab (antibody directed against VEGF) showed a synergistic antitumor activity in glioblastoma xenograft models [28]. A study by Mabjeesh *et al*, also identified a novel

antiangiogenic compound (2ME2), which induced downregulation of HIF-1 at a post-transcriptional level [21]. The data showed a significant antitumor effect, given HIF-1 induced down-regulation of VEGF. This rational provided evidence that dually targeting HIF-1 and other indirect pathways may be a robust strategy to overcome off-target therapy, and translate HIF-1 directed therapies to clinical development. Furthermore, a new interest has focused on designing multifunctional nanoparticles to deliver a combination of photosensitizers with hypoxia-activated prodrugs (HAPs) to tumor tissues, where induced hypoxia could activate HAPs, leading to a significant cell death and tumor growth inhibition [24, 25]. The latter approach will be discussed in more details in section 2.2. of this review.

2.1. HIF-1 inhibitors

The active role of hypoxia and HIF molecule has positioned them as novel therapeutic targets for cancer therapy [17, 26]. Classical approaches to overcome low oxygenation levels at the tumor site was carried out using hyperbaric chambers. Such intervention was proven to be unsuccessful in combination with radio and chemotherapy [14]. Therefore, new approaches have been introduced to target tumor hypoxia and small molecules that specifically target HIF-1, a master transcriptional factor that regulates cancer progression and development, are highly attractive. Existing therapeutic options, consider HIF-1 as a new gold standard target to treat cancer. Several small inhibitors have been developed and can inhibit HIF-1's activity by promoting HIF-1 protein degradation, or by blocking at least one of the following pathways: 1) HIF-1 mRNA expression; 2) HIF-1 protein translation; 3) HIF-1 DNA binding and 4) HIF-1 transcriptional activity [27, 28]. The demanding role and complex network underlying the HIF molecule indirect inhibitors have been thoroughly reviewed [29]. These include molecules that target upstream or cross-talked pathways with the HIF target (e.g. inhibitors of VEGF, mTOR, epithelial growth factor receptor (EGFR), topoisomerase I and II, PI3/ AKT/MAPK pathways). Despite the discovery and development of several therapies targeting HIF-1 or HIF-1 pathways, only a few have progressed into clinical development (Table 1). There have been no approved drugs that directly inhibit HIF-1, and many small molecule inhibitors have shown a high rate of late-stage clinical failure. This is attributed to the high redundancy and complexity of the tumor microenvironment. Furthermore, the desired effects of indirect inhibitors may be difficult to separate, as many different signaling pathways are linked to HIF-1 induced tumorigenesis, leading to the existence of off-target effects that are likely to be less predictable [30]. However, as mentioned above, recent studies have shown that dually targeting HIF-1 and other indirect pathways may be a strong strategy to overcome off-target therapy and translate HIF-1 directed therapies to clinical development [21, 22, 31].

Although there is a much better understanding of the pathway today than in the early 1990s when HIF-1 was discovered, its dual role in promoting cell survival and inducing apoptosis has caused controversy among researchers as to whether it should be considered as a therapeutic target

[32]. Nonetheless, selective gene therapy can be achieved by designing therapeutic genes, which are controlled by response to binding of HIF-1 to HREs (hypoxia responsive elements). Shibata *et al* (2

Table 1. Most promising HIF-1 inhibitors in preclinical and clinical trials.

Drug	*Method of action*	*Stage*	*Chemical class*	*Cancer type*	*Limitations*	*Main observations/conclusions*	*Reference(s)*
17-AAG (tanespimycin) and 17-DMAG (alvespimycin)	Bind to chaperone protein Hsp90, inducing proteosome degradation of HIF-1α	Phase I and II	Second generation benzoquinone ansamycin antibiotics	Prostate, hepatic, colon, ovarian, breast and glioma	Off target effects, poor bioavailability and solubility	First generation: galdanamycin - blocked HIF-1α protein expression in both serum and serum free normaxic and hypoxic conditions. Failed to enter clinic due to poor pharmacological properties and hepatoxicity in animal models; 17-AAG was the first-in-dass Hsp90 inhibitor to enter Phase I trials. Showed poor results in Phase II trials: 1) effect on RAF kinase expression were short-lived, and no objective anti-melanoma responses were seen; 2) At the dose and schedule used in this trial, 17-AAG did not achieve objective response in the treatment of clear cell or papillary renal cell carcinoma patients. 17-DMAG, an orally available agent, has shown promise in the clinic, with success in Phase I trials.	[22, 27, 31, 33-35]
YC-1	Inhibition of HIF-1α accumulation, possibly through an independent ubiquitin/proteosome pathway	Preclinical	Benzylindazole	Multiple tumors	Unknown mechanism of action	Interferes with mitogen pathways and thus affecting the translational process of HIF-1α; Down-regulates HIF-1α mRNA translation; First study to show hypoxia and mitogen-dependent inhibition of both HIF-1α and HIF-1β accumulation.	[36]
EZN-2968	Oligonucleotide targeting HIF-1 antisense mRNA	Phase I	Anti-sense oligonucleotide	Metastatic liver cancer	siRNA delivery	*In vitro* studies in prostate cancer and glioblastoma showed	[37-40]

						a potent selective and durable antagonism (IC50 1-5nM), under hypoxia and normaxia; VEGF levels are reduced, alongside tumor reduction in xenograft models; The activity of EZN-2698 in clinical trials at the doses tested was minimal and the study was halted.	
AFP464	HIF-1 mRNA interference	Phase I	Aminoflavone	Advanced metastatic solid tumors	Mechanism of action is unknown	Modulates mRNA expression of HIF-1α (full mechanism still not elucidated) and dimerizes with HIF-1β; Acts as a ligand for the aryl-hydrocarbon receptor, but inhibition of HIF-1 accumulation is independent on the receptor, although some data indicates otherwise; Clinical results have been observed for breast, renal and ovarian cancer, with maximum tolerability assessed.	[37, 41]
Topotecan	Inhibits HIF-1 translation by targeting topoisomerase I	Pilot study	Fluorine-19-Fluoroded	Solid metastatic tumors	Short half-life	FDA approved, as a second line of therapy for patients with small cell lung or ovarian cancer; Inhibits HIF-1 translation in a DNA damage independent mechanism, but acts as a poison for topoisomerase I; Results in a mouse xenograft model showed inhibition of HIF-1 expression, angiogenesis and tumor growth. An ongoing pilot study is in order.	[22, 42, 43]
EZN-2208	Inhibits HIF-1 translation by targeting topoisomerase I	Phase I & II	Pegylated form of Irinotecon (camptothecin)	Metastatic colorectal cancer	Off-target effects	Showed remarkable antitumor activity in pre-clinical trials for solid tumors and lymphoma, due to high solubility, accumulation and circulation time; In combination therapy trials with cetuximab, both drugs	[44, 45]

						were well tolerated, but no differences were obtained in the overall survival and progression, when comparing mono and combined therapy.	
Digoxin	Inhibits HIF-1α translation	Phase II	Cardiac glycoside	Operable breast cancer	Narrow therapeutic window	Digoxin and other cardiac glycosides blocked expression of HIF-1α even in the presence of the PHD-VDL pathway; Therapeutic action occurs even under normaxia; A 73% inhibition of HIF-1α translation occurs after treatment with Digoxin, while only 17% of overall accumulate protein is degraded; Blockage of expression occurs for P493 and PC3 cells, both *in vitro* and *in vivo*.	[46]
PX-478	Blocks deubiquitination and reduces transcription/ translation	Phase I	phenyl propionic acid *N*-oxide dihydrochloride	Advanced solid tumors and lymphoma	Off-target effects	Reduced tumor volume by 87% and mediastinal metastasis contributing to overall prolonged survival of NSCLC models; Highly active against orthotopic models of human lung cancer; The drug was also observed to decrease overall total protein synthesis, with a more pronounced effect in hypoxia in prostate cancer cells.	[47, 48]

proved this concept through the development of vectors containing a bacterial nitroreductase gene, with a responsive domain to VEGF [49]. This system was used to selectively activate an anticancer prodrug (CB1954). In a similar approach, the HRE vector was for human flavoprotein cytochrome P450 reductase, which was also used to selectively activate RSU1069 prodrug [50]. Both studies showed increased cytotoxicity in hypoxia, compared to normaxia.

Hypoxia-driven triple suicide genes have also been explored to increase the cytotoxicity of ganciclovir (GCV) and 5-fluorocytosine (5-FC) both *in vitro* and *in vivo* [51]. Additionally, antisense or small interfering RNA (siRNA) plasmids injected in hypoxic tumors has shown to eradicate EL-4 thymic lymphoma in combination with angiostatin [52]. Although these systems have shown an interesting outcome, off-target effects are still an issue, if injected intravenously. The use of drug carriers could further improve the efficacy and safety of gene delivery to the hypoxic tumor microenvironment.

2.2. Hypoxia-activated prodrugs

The concept of bio-reductive prodrugs (also known as hypoxia-activated prodrugs) has arisen, and the use of drugs that are non-toxic until they are reduced under low oxygen levels has opened the door to specific systemic treatments of solid and metastatic tumors [53]. Hypoxia-activated prodrugs are defined as cytotoxins that are metabolized by various endogenous reducing enzymes (oxireductases such as NAD(P)H, cytochrome P450) and quinone oxidoreductase (NQO1 in a one-electron or two-electron catalysis). The one step electron reduction is a selective process, as it composes a reversible step that generates a prodrug free radical that can easily back-oxidized to its original compound if oxygen is present, conferring specificity to highly hypoxic regions. Contrary to this, the two-electron reduction fails to produce an oxygen sensitive intermediate, being highly toxic, even for tissues in normaxia. The choice of which reduction occurs depends on the structure of the compound itself and the differential expression of reducing enzymes in tissues [20, 54]. The different classes of hypoxia-activated prodrugs are: (nitro (hetero) cyclic compounds; aromatic N-oxides; aliphatic N-oxides; quinones and metal complexes. These drugs are thoroughly summarized in Table 2, with the indication of their overall success in clinical trials. Although the values of these prodrugs are highly dependent on their relative metabolism and penetration, many have progressed significantly and showed promising applications as selective hypoxic cytotoxins [53].

Tirapazamine (TPZ) is the most clinically advanced hypoxia- activated prodrug [45]. Early clinical studies for non-small cell lung cancer (NSCLC), head and neck-cancer, metastatic melanoma (Phase I and II) and other solid tumors, showed enhanced tumor inhibition for this agent [55, 56]. This was achieved by co-administering TPZ with radiosensitizing and platinum agents. Furthermore, a dose-defining study of TPZ combined with embolization in liver cancer is now recruiting [57] . However, unexpectedly, a recent

randomized clinical trial combining radiation and cisplatin, with TPZ, for cervical cancer (Phase III) [58], showed that the combined

Table 2. Bioreductive prodrugs for hypoxia.

Prodrug	*Method of action*	*Stage*	*Chemical class*	*Cancer type*	*Limitations*	*Main observations/conclusions*	*Reference(s)*
Tirapazamine	Complex DNA damage	Phase III	Aromatic N-oxide	Head and neck (phase I and II) and cervix (Phase III)	low drug penetration and accumulation in tumor tissues	Potentiates antitumor effects after radiation sensitization; Phase I and II clinical trials, showed overall promising effects of TPZ in combination with radiation and cisplatin; CATAPULT 1 clinical trial for NSCLC showed an increase to 33.9% survival rate over 1 year Recent Phase II randomized clinical trials for head and neck cancer, showed no overall significant effect on response rate and survival; Review of populations used may be in order for correct assessment of the activity of the drug.	[55, 58-60]
SN30000 ***(CEN-209)***	Complex DNA damage	Preclinical	Aromatic N-oxide (analogue of TPZ)	Multiple tumors	Scarce literature reports	Has undergone extensive optimization to overcome low drug penetration and accumulation; Scheduled to enter Phase I clinical trials.	[61]
Apaziquone (E09)	DNA interstrand crosslinking	Phase III	Quinone	Bladder	Poor pharmacokinetic properties;	Results considering recurrence rates showed encouraging data (only 34.7 recurrence over 12 months and 48% after 18 months); No activity was seen against a wide range of tumors in subsequent phase II trials, probably due to the drug limitations; A promising outcome was seen, after administration of the drug directly into the urinary bladder.	[62-64]

Evofosfamide (TH-302)	DNA interstrand crosslinking	Phase II/III	Nitroimidazoles	Multiple tumors	Optimization of therapeutic regimen of combined therapy. One drug may compromise the action of another	Shows promise in terms of balance between stability and the reduction/oxidation equilibrium; Preclinical studies indicated a potent broad activity in various ectopic, orthotopic and metastatic models, for both mono and combined therapy; Phase I trials indicated good tolerance of the drug and partial responses in patients with metastatic lung cancer and melanoma; Phase II trials showed efficacy in combined therapy with gemcitabine and doxorubicin, under normoxia and hypoxia and ongoing phase III clinical trials are in order.	[65-68]
PR-104	DNA interstrand crosslinking	Phase II	Nitrobenzamine mustard	Leukaemia	Activation by aerobic reductases reduces hypoxia selectivity	Showed 10 to 100 fold increased activity in hypoxia, in vitro, for a broad range of cell lines, as well as a single agent efficiency in 6 of 8 xenograft models tested; Additive effect in combination therapy was observed, for both in vitro and human panels. Potentiation of drug may be due to a bystander effect caused by activation in oxygen present environments and hypoxia.	[69-73]
Banoxantrone (AQ4N)	DNA intercalator and topoisomerase II inhibition	Recent phase I/II	Aliphatic N-oxide	Multiple tumors	Lack of experimental data in patients, results may not be fully translated into therapeutics	Measurable decreases in hypoxic cells was observed and preclinical activity indicated accumulation of the drug in 24/30 tumor tissue samples; Radiation conditions, cisplatin and other combinatorial chemotherapy regimens have shown to have effect with this drug; AQ4 the active metabolite, after reduction, co-localizes with	[37, 74, 75]

						Glut-1 and hypoxic regions.	
SN-24771	Reduction of metal centers [Co (III) to Co (II)],	Pre-clinical	Metal complexes	Multiple tumors	No development for clinical use	This compound showed moderate activity in vitro, but failed to reproduce activity in in vivo hypoxia; It forms an unstable compound that releases its mustard ligand, destabilizing DNA; Other selective metal complexes have been reported, but improvement regarding this class of bioreductive drug is needed.	[27, 53]

treatment was not superior to cisplatin alone, since no change in the overall survival rate was observed. Although these results seem disappointing, one must take into account the small population size for this particular trial, and that the patients may constitute a non-selective hypoxia sub-population [60]. Therefore, we believe that it is essential to overlook apparent clinical failure and understand the overall limitations of these pro-drugs. This can be achieved by using *in vitro* systems that can accurately validate the therapeutic efficacy of these drugs. Furthermore, translation to clinical scenarios should be based on selecting hypoxia sub-populations. Evofosfamide (TH-302) is an investigational hypoxia-activated prodrug that releases the DNA alkylator bromo-isophosphoramide mustard under hypoxic conditions [66]. Phase I/II clinical trials have shown promising results for the drug as both a single agent and in combinatorial treatments with gemcitabine and nab-paclitaxel for pancreatic cancer [67, 68, 76]. A phase I/II Open-label Study of TH-302 and dexamethasone in subjects with relapsed/refractory multiple myeloma is now recruiting [77]. Phase II placebo-controlled study of TH-302 in combination with Pemetrexed in patients with non-squamous non-small cell lung cancer has started (NSCLC) [78]. PR-104 is a water soluble phosphate ester that has shown rapid hydrolysis *in vivo* with promising preclinical outcomes [79]. PR-104 is currently in Phase II trial for relapsed or refractory acute myeloid leukemia [71].

Although several hypoxia-activated prodrugs have progressed into clinical trials, their poor extravascular space penetration, short blood half-life, instability and poor balance between the reduction/oxidation equilibrium, have limited their accumulation in the target tissues [53]. A solution to overcome these limitations may underlie the development of novel carriers to deliver these drugs. Therefore, improving their cellular uptake, specificity, tissue penetration, pharmacokinetics and dynamics [80, 81]. From a clinical point of view, the standardization of treatment, dynamic interplay between hypoxia and other molecular determinants, as well as the poor definition of patient subgroups for hypoxia-targeted treatment, has caused limited positive clinical outcomes and warrants further evaluation [16, 82]. In support of these findings, recent studies have utilised nanocarriers to improve HAPs poor *in vivo* distribution, and to offer a combinatory treatment with photosensitizers. TPZ is well known for its moderate to severe hypoxia activation, but other HAPs seem to have a narrow window of action. The use of photodynamic therapy (PDT) could create a more selective hypoxia environment through photosensitization, with increased synergism with HAPs. Shi *et al* and his co-workers presented an interesting piece of work where they validated that photosensitizers can indeed produce a strong enough hypoxia environment that can potentiate the action of HAPs [25]. In this paper, double layered silica-shell nanoparticles were developed, which were capable of co-delivering a photosensitizer (UC/PS) and TPZ. The drug-loaded nanoparticles (TPZ-UC/PS) showed excellent biocompatibility and sufficient oxygen depletion by the photosensitizer, resulting in a significant reduction in cell viability. Furthermore, they showed a remarkable reduction in tumor volume after near-infrared irradiation

(NIR). These combinatorial synergistic systems could offer a potential treatment for patients with large deep-seated tumors. In the same line of work, a mesoporous silica-based theranostic platform was also developed for synergistic PDT and hypoxia cancer therapy [24]. CD44- targeted system was comprised of a layer-by-layer silica nanoparticles that were used as a drug reservoir for TPZ, with an assembled supramolecular photosensitizer ($TPPS_4$), coordinated with the paramagnetic agent gadolinium-III for tumor-targeted diagnosis and treatment. Interestingly, PDT-induced apoptosis was confirmed by enhanced ROS production and the system showed a remarkable synergistic effect under 21% oxygen conditions, where the most significant reduction in tumor volumes was observed in mice co-treated with PDT and TPZ nanoparticles. These two studies confirmed that PDT could be used as a successful approach to activate HAPs, and to induce a synergistic effect *in vivo* models.

3. Tumor-associated macrophages (TAMs)

3.1. Macrophages in the tumor microenvironment

The presence of dendritic cells, fibroblasts, endothelial cells, monocytes and macrophages in the tumor microenvironment has been repeatedly linked to tumor progression [83]. The high infiltration of the immune cells in the tumor microenvironment, essentially in the hypoxic cores of tumors, has been correlated with a negative clinical outcome concerning patient's survival.

Monocytes are the primary moderate phagocytic cell type that contain primary lysosomes and have an overall pro-inflammatory effect. These cells circulate for about 1 to 3 days and afterward move to tissues where they mature into more active phagocytic cells, the macrophages (MACs) [84]. These cells are native residents of organs such as the liver, lung, spleen, lymphatic nodes. They have an important immunosurveillance role, mainly responsible for tissue repair, wound healing and defense against pathogens, including tumor cells [85]. However, a differential MAC programming in cancer progression has been elucidated, and much needs to be taken into account when describing the primary role of these cells in the tumor microenvironments [86]. MACs are particularly abundant and present at all stages of tumor progression. Their role in the tumor environment has reached a concept of 'friend or foe', recognizing that a well- functioned immune system should destroy tumor cells [87]. Nonetheless, many cancer cells escape the tight immune surveillance and redirect immune cells to their advantage. This escape is due to the development of deficiencies in antigen processing and presentation in several MAC pathways, and the consequent production of immune suppressive cytokines [88]. Tumor cells are known to secrete different cytokines, which control the balance between pro-inflammatory and anti-inflammatory components that in turn are responsible for this dual shift in MAC genomics and proteomics. Chemotactic cytokines, such as CXCL1, CXCL8, CXCL12, CXCL13, CCL5, are used to attract circulating monocytes to the tumor microenvironment [89-91]. These residing monocytes can then differentiate into MACs and initiate an inflammatory imbalance

that causes a vicious cycle in which MACs initially control tumor development, but once established, are educated to become protumoral [23, 92].

3.2. Differential polarization and the relevance of the M2 protumoral effect

Two different polarization status have been reported amongst the MACs population [93]. The M1 phenotype (classically activated form) is responsible for host defense. When activated by Interferon gamma (IFN- γ) and/or lipopolysaccharides (LPS), M1 MACs produce large amounts of pro-inflammatory cytokines, such as oxygen species (e.g. nitric oxide), high levels of MHC (Major Histocompatibility Complex) molecules, interleukin 12 (IL-12) and low levels of IL-10. These characteristics make them potent killers of pathogens and tumor cells. On the other hand, the M2 subtype (alternatively activated form) responds to stimulus from IL-4/IL-13, IL-10, Toll-like receptors (TLR), glucocorticoids, allowing the development of MACs involved in tissue remodeling, angiogenesis, immune-regulation (M2b or M2c) and tumor development (M2a). These cells produce high amounts of IL-10, tumor necrosis factor (TNFα) and arginase-1. They over-express scavenger receptors, mannose receptors and exhibit anti-inflammatory activity [94, 95].

The definition of M1 and M2 is not a 'black and white' concept. M2 type MACs are not simply defined by their location, but also by the stimuli they receive in the specific environments, in which they reside. Although these extreme forms of polarization are seductive, it seems that the multiple tumor-associated macrophages (TAMs) phenotype is dependent on the stimuli received in the complex network of signaling in the tumor microenvironment, as well as the tumor itself [92]. TAMs have been defined to closely resemble the M2 phenotype, and to constitute up to 80% of the tumor mass [93]. They are highly accumulated in poorly vascularized, hypoxic and necrotic areas, contributing to a poor prognosis and exerting a protumoral effect in many types of solid tumors [96, 97].

Over forty years ago, a study by Fidler in 1974 [98], demonstrated that intravenous injection of specifically activated MACs (M1 phenotype) inhibited lung tumor metastasis. On the other hand, Gorelik *et al* [99] showed that injecting M2 MACs intravenously in mice increased the development of lung cancer nodules. In the last two decades, studies have shown that co-culturing tumor cells *in vitro* with M2 MACs, even in the absence of a direct contact, increased tumor angiogenesis, progression and invasiveness [100-103]. These results highlight the different roles that polarized MACs play in cancer progression.

3.3. M2 MACs as promising therapeutic targets for cancer

Several studies have shown that MACs play a fundamental role in tumor development, and, therefore, remain a respectable therapeutic target in cancer therapy [104]. M2 MACs overexpress different types of receptors, which makes them excellent targets for drug delivery. A summary of the different targeted delivery systems to these MACs is shown in Table 3.

Table 3. Successful targeting approaches for TAMs.

Targeting receptor	*Targeting ligand*	*Therapeutic agent*	*Delivery system*	*Tumor model*	*Outcomes*	*Reference(s)*
Mannose (CD206$^+$ human, CD205$^+$ murine)	Glucomannan	Alendronate	Conjugated polysaccharide vehicle	Mouse macrophage cell line Raw 264.7, mouse sarcoma cells, human umbilical vein endothelial cells and human lung carcinoma A549 cell line; Murine sarcoma S180 *in vivo* model	Both ALN and ALN-BSP (100 mM) induced RAW 264.7 apoptosis; Fluorescent images and flow cytometry analysis suggested that ALN-BSP was preferentially taken up by Raw 264.7 macrophages (33.1%), in comparison to HUVECs (11.2%) and A549 (2.36%); ALN-BSP was found to inhibit angiogenesis (decreased the level of VEGF by 83.9%) and expression of MMP (by 65.3%).	[105]
	Anti-MMR specific nanobodies	N/A	Nanobody based	C57BL/6 MMR-deficient, CCR2-deficient, and MMTV-PyMT mice; Balb/c mammary adenocarcinoma models	*In vivo* injection of 99mTc-labeled α-MMR Nb tumor bearing mice confirmed its specificity to TAM's; Co-staining for the nanobody and hypoxic showed that α-MMR Nbs can target hypoxic tumor regions *in vivo*.	[106]
	Alkyne functionalized mannose	siRNA	Polymeric micelles (pH sensitive hemolysis)	Murine BMDM's; Human macrophages (THP-1); Breast cancer cell lines (MDA-MB-231 and MDA-MB-468)	The tri-polymer efficiently complexed siRNA and protected the genetic cargo. Macrophages presented a 26-fold increase uptake of selected micelles, comparative to tumor cells; Flow cytometry results showed that mannose targeting significantly increased the rate of delivery of siRNA into macrophages and generated a robust knockdown of the model gene.	[107]

	O-stearoyl mannose	N/A	PEG-sheddable mannose modified PLGA nanoparticles	J774A.1 murine macrophages; B16-mouse melanoma tumors in C57BL/6 mice	Pegylation of M-NP significantly decreased the cellular uptake by 75%; After injection of the nanoparticles into C57BL/6 mice the PEG shielding was important to minimize uptake by off-targets; Injection of modified and unmodified particles in mice, showed higher tumor accumulation, as PEG shielding enhanced circulation time. Acid- sensitive PEG shedding, allowed for a specific and localized binding and efficient uptake by M2 type MACs.	[108]
CSF-1R	Anti-CSF-1R (RG7155)	N/A	N/A	TAMs *in vitro*, in *in vivo* animal models (male cynomolgus monkeys, Female C57BL/6N mice) and in RG7155-treated Dt-GCT patients	G7155 potently inhibited the viability of CSF-1-differentiated macrophages with an IC50 of 0.3 nM; RG7155 mediated rapid elimination of alternatively activated MACs ($CD14^+CD16^+$), but not of classical MACs ($CD14^+CD16^-$), in cynomolgus monkeys; Clinical activity correlated with a significant reduction of $CD68^+/CD163^+$ macrophages and of $CSF\text{-}1R^+$ cells in matching tumor biopsies and the dramatic TAM reduction was independent of the degree of basal macrophage infiltrate.	[109]
Legumain	Legumain	N/A	DNA construct encoding legumain	Murine macrophage cell line RAW 264.7, murine 4T1 breast carcinoma cells and female BALB/c and C57BL/6 mice	Lung metastasis's weights were determined at 24 days or 30 days after. Differences between the 2 control groups (PBS and/or empty vector) and the treatment group were statistically significant; $^{**}P < 0.005$. It was shown that, T cell response abrogated M2 type macrophages after application of the legumain-based	[110]

					DNA vaccine, which in turn effectively inhibited spontaneous 4T1 breast cancer metastases. In murine models of metastatic breast colon, and non–small cell lung cancers, 75% of vaccinated mice survived lethal tumor cell challenges and 62% were completely free of metastasis.	
Macrophage galactose-type lectin (Mg1)	Galactose	CpG ODN Anti-IL-10 ODN and IL-10RA ODN	Cationic dextran based nano-complex	Hepa-1-6 mouse hepatoma cell line and female ICR mice as allograft model for liver cancer	Compared to control, FITC-ODN monitoring confirmed efficient transfection of and blockage of Mg1 *in vivo*, while the pH sensitive moiety conferred controlled and accurate release of the ODN from the complexes; The nano-complex reversed TAM phenotype by decreasing M2-specific genes: Arg-1, Ym1, Mg1, Mg2 and IL-10. Additionally, this system showed enhanced anti-tumor activity in an allograft model, as mean tumor weights were reduced and histological analysis of liver, showed large necrotic areas.	[111]
IL-4Rα	Peptide (CRKRLDRNC)	N/A	N/A	CHO-K1 cell line and C567BL/6 male mice	The peptide did not home to other organs and co-localization of the peptide with macrophages was observed; The peptide bound strongly to CHO-K1 cells overexpressing IL-4Rα, compared to control. CRKRLDRNC bound both human and murine cells, presenting cross-reactivity of species.	[112]
	Anti-IL4 aptamer	N/A	N/A	4T1 and MSC2 cell lines BALB/C mice	FACS analysis showed that the aptamer preferentially bound its ligand *in vivo*, with enhanced targeting of TAMs in tumor bearing mice; the aptamer recognized and	[113]

bound both murine and human MACs;

It may also trigger biological activity, possibly through a pro-apoptotic activity on TAMs, as tumor growth was inhibited in mice treated with the aptamer and a dramatic decrease in secondary metastasis was also observed.

The principal mechanisms of MAC-based anticancer therapy rely on 1) inhibiting monocyte maturation in resident tumors; 2) depleting M2 type MACs; and 3) shifting polarization to an M1 phenotype with the pro-inflammatory response.

Therapeutic agents have been developed to target TAMs. For instance, trabectedin is an anticancer drug, licensed to treat advanced soft tissue sarcoma and recurrent ovarian cancer. It works by inhibiting macrophage colony-stimulating factor (M-CSF)-driven differentiation of monocytes into MACs, and blocking the production of CCL2, IL-6, and VEGF, causing a subsequent inhibition of tumor progression [114-117]. Prednisolone is a second class of drug (glucocorticoid) that inhibits monocyte differentiation. Systemic administration of prednisolone phosphate (PLP) encapsulated in long-circulating liposomes (LCL-PLP) significantly reduced M2 MAC levels in the tumor tissue, decreased the production of chemoattractants involved in the infiltration of monocytes into tumor microenvironment, and lowered the production of angiogenic factors by M2 MACs [118-120]. Metalloproteinase-9 (MMP-9) is a protein that is expressed by M2 MACs, which in turn triggers the release of VEGF, a factor important in the angiogenesis of tumors. Inhibiting tumor metalloproteinase activity, and diminishing the association of VEGF with its tyrosine kinase receptors on proliferating endothelial cells, may be an effective strategy to inhibit tumor growth. Clodronate, a bisphosphonate drug that blocks the activity of MMP-9 in TAMs. A liposome-based carriers containing clodronate (LIP-CLOD) selectively targeted MMP-9 following phagocytosis and intracellular drug release. MACs phagocytosed the LIP-CLO, which were subsequently degraded by the lysosomal phospholipases releasing the clodronate into the cell and inducing apoptosis, and reduced blood vessels formation [121].

The third mechanism in MAC-based anticancer therapy relies on shifting M2 polarization to M1 MACs [122]. TAMs express primarily M2-like phenotype, but can polarize to a classical M1-like phenotype, due to their high plasticity. For M1 polarization to occur two specific pathways have to be targeted, nuclear factor-κB (NF-κB) and the signaling transducer and activator of transcription (STAT) pathway. Toll-like receptors (TLR) are also expressed on MACs and is considered essential for the activation of NF- κB pathway, causing a reverse in MAC phenotype [88]. TLR agonists and inducers of NF- κB pathway have been exploited, causing a significant decrease in tumor growth [123]. Murphy *et al* demonstrated that Azitromycin-treated J447 mouse MAC cells, produced lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines[124]. This clearly demonstrated the capacity of this antibiotic to shift the M1 polarization to M2. This study contributed to a better understanding of MAC function and polarizability in early inflammation. However, further studies to evaluate the clinical application, are still needed.

Gene therapy has also been explored to re-educate TAMs at tumor tissues. Adenoviral transduced MACs with IL-12 were used to treat orthotopic 178-2 BMA mouse prostate cancer model. The systemic administration of over-expressing IL-12 M1 MACs significantly reduced the growth of primary tumor and its metastasis in mice [97, 125]. Lately, nano-carriers containing retinoic acid succeed in reverting M2 phenotype into M1. Flow cytometry and fluorescent microscopy analysis demonstrated that these nanocarriers were efficiently taken up by MACs, following systemic administration and remained engulfed in TAMs for 7 days [126]. Another study by Lobenberg group reported the capacity of doxorubicin-loaded nanoparticles to activate MACs (shifting naïve MACs to a M1 phenotype) after phagocytosis, which led to a significant cell death in cancer cells [127].

4. The link between hypoxia and TAMs: the promising role of targeted drug delivery systems

Evidence has connected tumor aggressiveness and poor patient survival to the hypoxic regions of tumors. TAMs infiltrate the hypoxic perinecrotic areas of the tumors, due to the increased number of cellular debris in those regions. Therefore, promote immune suppression, angiogenesis, lymphogenesis and matrix remodelling [9, 10]. Such correlation has been observed in patients diagnosed with breast, prostate, ovarian and cervical cancer [128, 129]. TAMs have been able to modulate the transcription factor HIF-1, several survival pathways, such as phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathway, as well as regulate tumor angiogenesis and metastasis [19, 20]. Recent reports have suggested that HIF-1 is overexpressed by TAMs, thus completing the vicious cycle that promotes cancer survival, progression and resistance to therapy [15] (Fig. 2). HIF-1 transcription factor not only modulates the expression of cancer-related genes in TAMs, but also provokes a metabolic shift in these immune cells, thriving the tumor development under nutrient and oxygen deprivation [130].

Individual and combinatorial systems exploiting TAMs and tumor hypoxia have been proposed. Hypoxia-responsive polymeric micelles showed a superior therapeutic efficacy in mouse cancer models, following systemic administration. This effective anti-tumor activity *in vivo*, was due to the high selectivity and fast intracellular release of doxorubicin from the responsive nanoparticles, under low oxygen levels. Quantitative analysis showed a 4-fold increase in the accumulation of responsive hypoxia micelles in tumor tissues, compared to normal tissues, which resulted in a slower tumor growth [131]. Another approach to target tumor hypoxia was demonstrated by Wang *et al* and his team by developing micellar-based nanoparticles for the delivery of HIF-1α siRNA (EZN-2968). These nanoparticles showed specific gene knockdown both *in vitro* hypoxic mimicking cultures and *in vivo*

hypoxic tumor models for prostate cancer. HIF gene silencing inhibited cell migration and angiogenesis (reduction of VEGF levels) and increased the sensitivity of cancer cells to doxorubicin [132]. Recently, Quan *et al* reported the development of galactose-based thermosensitive nanogels as a theranostic system for hypoxic hepatocellular carcinoma [133]. The system was used to deliver iodoazomycin arabinofuranoside (IAZA), a radiosensitizer and a hypoxic imaging agent, to hypoxic hepatocellular carcinoma cells. IAZA-loaded nanogels showed a higher sensitization enhancement ratio, compared to IAZA alone. These results indicated that using nanogel particles could be a potentially useful approach to enhance the therapeutic efficacy of hypoxia radiosensitizers in tumor tissues.

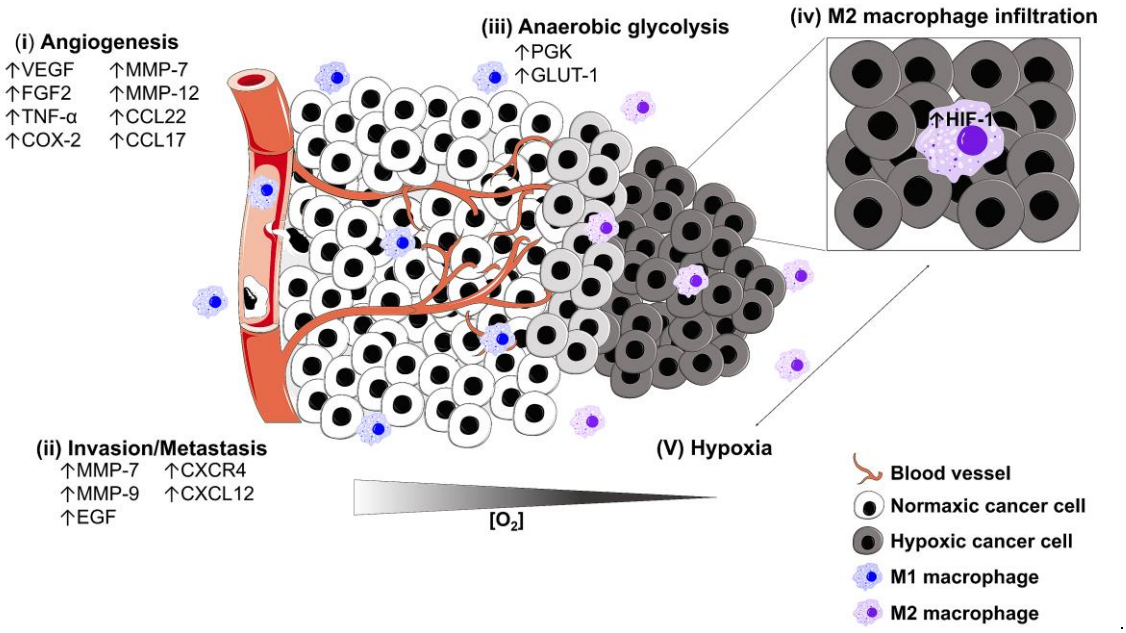

Fig 2. The hypoxic tumor environment and its role in oncogenesis. Deprivation of oxygen in the tumor core has been linked to tumor development and poor prognosis. Upregulation of HIF-1 has shown to enhance the expression of many cancer markers related to: (i) tumor angiogenesis, (ii) invasion and metastasis, (iii) metabolism shift and (iv) infiltration of M2 macrophages in the (v) hypoxic regions of tumors. The futile cycle created by the positive feedback of negative prognosis biomarkers in cancer are responsible for tumor survival and resistance to therapy.

CCL17, Chemokine (C-C Motif) Ligand 17; **CCL22**, Chemokine (C-C Motif) Ligand 22; **COX-2**, cyclooxygenase-2; **CXCL12,** Chemokine (C-X-C Motif) Ligand 12; **CXCR4**, Chemokine (C-X-C Motif) Receptor 4; **EGF**, Epidermal growth factor; **FGF2**, fibroblast growth factor 2; **HIF-1**, Hypoxia inducible factor 1; GLUT-1, Glucose transporter 1; **MMP-7**, Matrix metalloproteinase 7; **MMP-9**, Matrix metalloproteinase 9; **MMP-12,** Matrix metalloproteinase 12; **PGK**, Phosphoglycerate Kinase 1; **TNF-α**, Tumor necrosis factor alpha; **VEGF**, Vascular endothelial growth factor.

Lately, trigger responsive drug delivery nanocarriers have been proposed to target the acidic tumor microenvironment that usually associated with hypoxia [11]. For example, Poon *et al*

developed pH-responsive sheddable nanoparticles, consisting of multiple layers, of which neutral layers shed in response to the acidic environment, exposing the charged nanoparticle surface to be easily taken up by tumor cells, while sparing healthy tissues [134]. In another study, Meng *et al* successfully developed pH-responsive nanovalves [135]. The porous mesoporous silica nanospheres were loaded with the drug and the pores were plugged with β-cyclodextrin. The interaction between the β-cyclodextrin and the stalk was dependent on the pH level, eventually facilitating diffusion of the drug from nanopores in the hypoxic tissues. The latter strategy was further improved by Dong *et al* who successfully developed pH-sensitive polymeric-coated porous silica nanoparticles, by using polyethylene imine (PEI) and PEI–PEG co-polymers. Both polymers improved the biodistribution of silica particles *in vivo*, where a superior tumor accumulation was observed with sterically stabilised nanoparticles [136]. More interestingly, photodynamic therapy (PDT) has been exploited to facilitate hypoxia generation, triggering drug release from hypoxia-responsive nanocarriers [137]. In this work, a ROS-generating and hypoxia-sensitive 2-nitroimidazole-grafted conjugated polymer (CP-NI) was synthesized, and doxorubicin (Dox) was successfully encapsulated within these polymeric nanoparticles. These multifunctional nanoparticles generate ROS upon near-infrared irradiation (NIR), leading to a rapid oxygen consumption within the tumor tissues, resulting in an efficient drug release from the nanocarriers. Such system showed promising *in vitro* and *in vivo* results, where mice treated with CP-NI-Dox in combination with NIR irradiation showed a complete tumor growth inhibition, with high levels of apoptosis as confirmed by TUNEL assay. Combining PDT with HAPs has been also considered as a promising approach to enhance the therapeutic efficacy of anti-cancer therapy, as discussed in section 2.2.

The concept of using MACs as 'trojan horses' has proven to be an interesting approach in cancer therapy [138, 139]. Choi *et al* developed MACs loaded with gold nano-shell particles, which efficiently targeted the hypoxic regions of T47D breast cancer spheroids *in vitro*, and successfully slowed down the spheroid growth in combination with NIR [140]. A second study by Hirschberg *et al* showed the infiltration of gold–silica nanoshells-loaded MACs into glioma tumor spheroids [141]. Similarly, small gold nanorod-laden MACs (sAuNRs-laden MACs) with a smaller diameter of ~7 nm were able to infiltrate inaccessible tumor hypoxic regions, enhancing photothermal tumor ablation. The data showed that sAuNRs had higher cellular uptake (52.5%) and lower cytotoxicity in MACs, compared to other gold-based particles. RAW264.7 macrophages incubated with AuNRs and irradiated with 808 nm NIR laser, showed a significant cell death (higher ratio of cell apoptosis (32.6% ± 0.87) compared to the control group. Interestingly, these results were translated *in vivo,* where 95% tumor growth inhibition was observed in HepG2 tumor-bearing nude mice, with no visible recurrence [142]. Another study by Choi *et al* reported the development of drug-loaded

MACs to treat cancer [143]. This novel therapeutic system was composed of mouse peritoneal MACs loaded with liposomal doxorubicin (macrophage-LP-Dox). Liposomal nanoparticles protected the MACs from the encapsulated doxorubicin *in vitro*, while delaying the tumor growth in animal models. These approaches highlight the potential use of MAC-based drug delivery systems to treat cancer. An Interesting work by Jiang *et al* using bone marrow derived MACs (BMDM) loaded with doxorubicin polymeric nanoparticles, demonstrated the deep penetration of doxorubicin into the tumor tissues, which was significantly higher in infrared irradiated tumors (IR) [144]. Such approach could be particularly suited to treating IR-induced recurrent tumors.

Model simulations have suggested that, compared with conventional chemotherapy, MAC-based therapy preferentially targets tumor cells and diminishes the hypoxic cells in the tumor core [145]. In agreement with these simulations, Griffiths *et al* developed a successful approach using a gene-dependent enzyme prodrug therapy (GDEPT), where MACs were transfected with adenoviral vectors that express cytochrome P450 [146]. The transfected MACs highly infiltrated tumor spheroids and enhanced the therapeutic activity of cyclophosphamide, a prodrug that induces tumor cell killing by the bystander effect. Treatment of spheroids with cyclophosphamide, after co-culture with transfected MACs, showed a significant reduction in spheroids volume. This approach became more promising after engineering adenoviral construct with a hypoxia-responsive promoter, where cytochrome P450 was only expressed in the hypoxic region of the tumor, highlighting the specificity of the treatment. Subsequent exposure of the tumor spheroids to cyclophosphamide and MACs transfected with a hypoxia-responsive construct showed a specific inhibition of cell proliferation under hypoxic conditions. Furthermore, Lewis' lab previously used MACs to efficiently deliver oncolytic viruses to hypoxic prostate cancer tissues *in vivo*. The engineered MACs were co-transduced with a hypoxia-regulated E1A/B construct and an E1A-dependent oncolytic adenovirus, whose proliferation was restricted to prostate tumor cells using prostate-specific promoter elements from the TARP, PSA, and PMSA genes [138]. A single systemic injection of the oncolytic virus-loaded MACs resulted in a marked inhibition of tumor growth and reduction of pulmonary metastases. Furthermore, such approach enhanced the therapeutic efficacy of chemo- and radiotherapy in prostate cancer models [147].This data demonstrated the high potential of multifunctional nanotherapeutics which target tumor hypoxia and MACs.

A second interesting approach was explored by Huang *et al* and co-workers [148, 149], who exploited the use of monocyte-mediated delivery of polymeric bubbles to potentiate re-oxygenation of tumor hypoxic areas, whilst enhancing the therapeutic efficacy of chemotherapy. In the first study, Huang *et al* presented an innovative strategy for overcoming the limited activity of photodynamic therapy (PDT) in hypoxic tumor tissues using bone marrow-derived monocytes as

cellular vehicles for co-transport of oxygen and a light activated photosensitizer, chlorin e6 (Ce6). Superparamagnetic iron oxide nanoparticles (SPION)/Ce6/oxygen-loaded polymer bubbles were internalized into tumortropic monocytes. In this study, results showed that intratumoral administration of therapeutic monocytes exhibited a superior activity in inhibiting tumor growth in Tramp-C1 tumor-bearing mice upon the treatment with magnetic field and light laser. Histological examinations of the tumor sections confirmed the successful cellular transport of the therapeutic payloads to tumor hypoxia. This study demonstrated that oxygen/therapeutic co-delivery via tumortropic monocytes enhances tumor penetration, relieves tumor hypoxia after external hyperthermia trigger, and sensitizes cells to PDT [148]. In the second study [149], the same principle was applied for the co-delivery of polymeric bubbles and doxorubicin-loaded polymeric vesicles. Here, focused ultrasound was applied to trigger the release of drug-loaded vesicles from the monocytes within the hypoxic cores of tumors following cell infiltration. Once again, *in vivo* and ex vivo fluorescence imaging showed an appreciable accumulation of the doxorubicin-loaded monocytes at the tumor site. With this, a high payload of drug was delivered deeper within the tumor mass, resulting in a pronounced cytotoxic effect. These studies clearly highlight the capability of MACs to deliver a wide range of therapeutics to deep tumour tissues, where the therapeutics efficacy was enhanced in combination with external triggers, such as laser irradiation, hyperthermia or ultrasound, which facilitated drug release from the nanocarriers.

Recently, manganese dioxide nanoparticles (MnO_2 NPs) have been used to target TAMs and enhance tumor oxygenation [150]. In this study, Song *et al* developed novel nanoparticles that target TAMs in the hypoxic regions of the tumor. The high reactivity of MnO_2 NPs toward hydrogen peroxide (H_2O_2) allowed the simultaneous production of O_2 and regulation of pH in the hypoxic core. These nanoparticles were further modified with mannose and hyaluronic acid (HA) to promote M2 targeting and TAMs repolarisation into M1 phenotype, respectively. The results showed higher association of the targeted particles to M2 mannose receptor *in vitro*, compared to untargeted particles (5-fold increase). Interestingly, targeted manganese dioxide particles showed a steady tumor uptake *in vivo* after intravenous injection and relatively low liver uptake. Flow cytometry assays, suggested the positively induced polarization of M2 to M1, with consequent production of high levels of H_2O_2 by these cells. The system improved the overall oxygenation and regulation of local hypoxic pH, in which the tumor showed 50.3% less tissue hypoxia, 49.3% decrease in the expression of HIF-1α, and 31.8% decrease in the expression of VEGF, 4 days after treatment. This study provided the opportunity to enhance chemotherapy response in 4T1 xenograft model by using combining Man-HA-MnO_2 NPs and doxorubicin, where a significant reduction in xenograft tumor growth was observed.

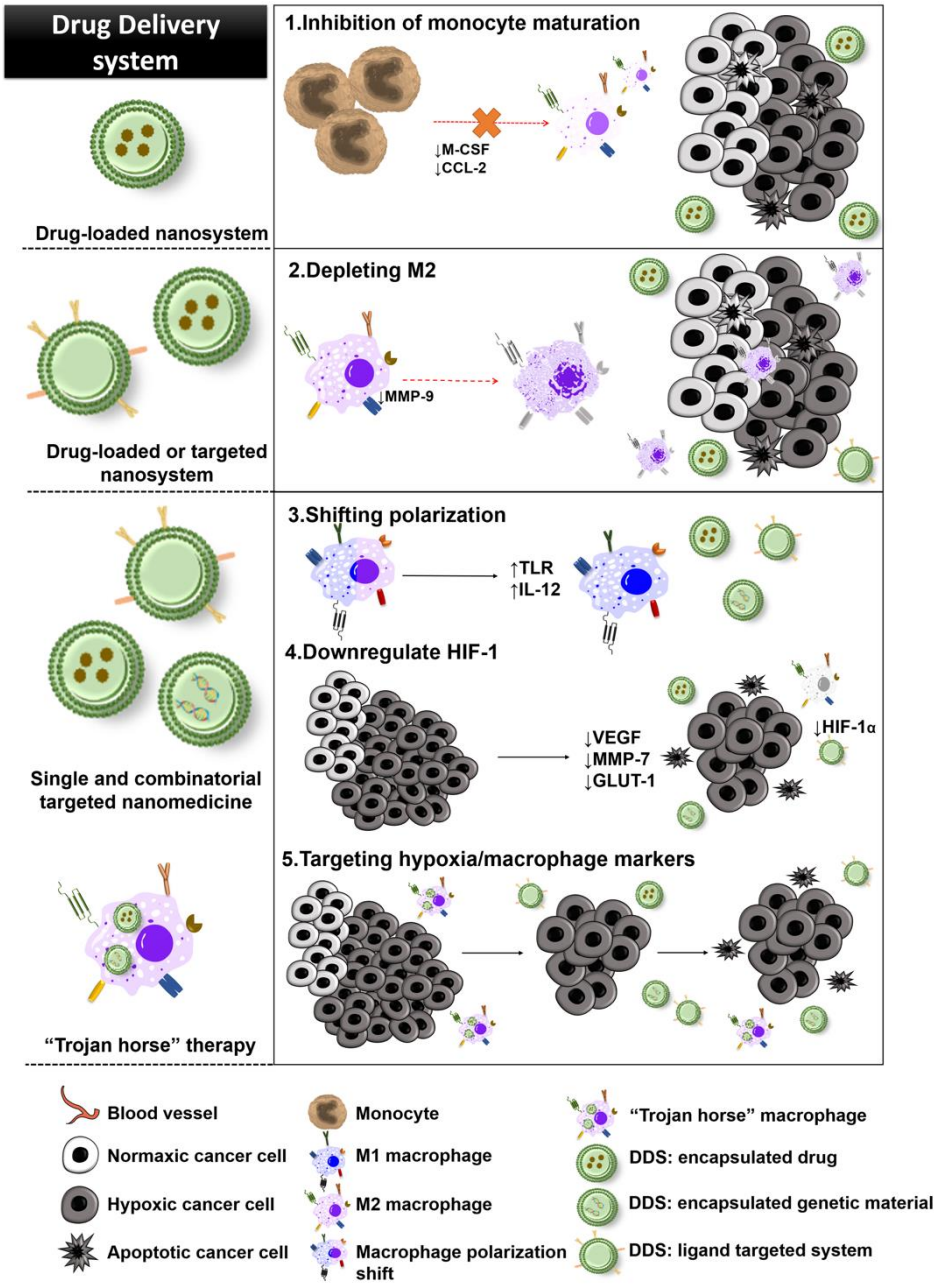

Fig 3. Nanoparticle-based therapeutics targeting TAMs and hypoxia. Drug delivery systems (DDS) have shown great promises to inhibit the effect of TAMs and hypoxia on cancer development. The main mechanisms that have been developed so far using nanoparticles to targets tumor microenvironment are: 1) inhibiting monocyte maturation, leading to a reduced accumulation of pro-tumor immune cells (M2) at the tumor site; 2) depleting the tumor from M2 macrophages; 3) shifting the polarization of M2 macrophages (pro-tumor state) to an anti-tumor M1 state; 4) downregulating HIF-1with specific inhibitors and ligands; and 5) using macrophages as trojan horses to deliver DDS to the hypoxic regions of the tumor.

CCL2, Chemokine (C-C Motif) Ligand 2; **DDS**, Drug delivery system; **HIF-1**, Hypoxia inducible factor 1; **GLUT-1**, Glucose transporter 1; **IL-12,** Interleukin 12; **M-CSF**, Macrophage colony stimulating factor; **MMP-7**, Matrix metalloproteinase 7; **MMP-9,** Matrix metalloproteinase 9; **TLR**, Toll-like receptor; **VEGF**, Vascular endothelial growth factor

In summary, we have demonstrated here that targeting hypoxia and TAMs is, indeed, a promising therapeutic targets for cancer therapy. Their mechanisms of action are summarized in Fig. 3. Nanomedicine is a smart strategy to reassess old drugs and improve their pharmacokinetics and tumor targeting. Therefore, we believe that special considerations should be taken into account to design a combinatory treatment, capable of exerting synergistic effects in the tumor microenvironment. Several studies reviewed here took the advantages of oxygen generation/consumption to manipulate the hypoxia environment and to sensitize cancer cells to different treatment strategies.

5. Clinical implications and future perspectives

Hypoxia has become one of the most attractive targets in cancer [11]. Many small molecule inhibitors, HAPs and hypoxia-responsive nano-systems have shown a great promise in cancer therapy. However, hypoxia-targeted therapy has shown controversial results in mouse models and humans. These high attrition rates of failure are attributed not only to the high complexity and redundancy of the tumor microenvironment, but also due to lack of veracity and fidelity in existing preclinical models and patient subsets. Therefore, substantial efforts have been made to develop more reliable *in vitro* methods that can then be successfully translated to *in vivo* set-up and in patients [30]. For instance, the potential of these novel therapies must be validated at early stages, by fully establishing cell culture models that include comprehensive immunohistochemistry (IHC) staining, western blot analysis, and mRNA expression of direct and indirect pathways linked to hypoxia [27]. Limited molecular biological testing has been carried out due to the lack of well-established robust biomarkers associated to hypoxia, and the nonexistence of *in vitro* models that can actively mimic the complex acidic, immune infiltrated tumor microenvironment, with heterogeneous expression of hypoxia-related genes. Nevertheless, biomimetic 'organ/tumor-on-a-chip' tools have presented themselves as promising new *in vitro* tools, that could bring together the advantages of tumor spheroids [151], with the aid of microfluidic systems that can offer information on tumor heterogeneity, interstitial flow, cell binding and nanoparticle/drug accumulation [152]. Despite these challenges, efforts have been made to create more realistic pre-clinical and clinical models that are essential to fully understand, validate and successfully predict the outcome of these targeted therapies. More importantly, many open discussions on the matter have highlighted the importance of patient pre-selection for hypoxia and tumor microenvironment targeted therapies [30]. Previous clinical set-ups have failed to show relevant positive outcomes, due to inadequate patients standardization. Many patient subsets did not show a relevant expression of HIF-1. Also, a good percentage of the patients showed mixtures of well-oxygenated and hypoxic tumors, or poorly established immune microenvironment that conferred reduced benefit of the applied treatment.

Clearly, there is still much to be done to perform adequate evaluation of these treatments, but recent efforts to establish xenograft models that constitutively represent HIF-1 expression encourage future studies and reliable clinical translation [143, 153]. Furthermore, the new interest to combine HAPs with drugs that are known to lowering tumor oxygen levels and potentiating tumour hypoxia, such as some VEGF inhibitors and photosensitizers, could enhance the therapeutic efficacy of the combinatory treatments, and overcome the oxygen tumor heterogeneity.

Similar to hypoxia, tumors have been known for their heterogeneity in blood supply, which results occasionally in inevitable disappointment once translated to clinical trials. This tumor heterogeneity makes it difficult to predict drug efficacy in tumor models, or to extrapolate the clinical efficacy of drugs in humans based on *in vivo* mice models. In support of this, Danhier *et al* has recently highlighted the need to reassess the concept of nanomedicine in a clinical aspect, mainly regarding its benefits when exploiting the enhanced permeation and retention (EPR) effect [154]. Danhier argues that although the EPR is probably one of the most cited and important concepts in nanomedicine, it is a heterogeneous process, highly variable between tumors and within the same tumor mass, and not the main attribute for the high success of novel nano-therapies. Nevertheless, as aforementioned, we highlight the importance of using more relevant models that can translate the results obtained in murine models to cancer patients. For example, patient-derived tumor explant (PDX) models, can alternatively provide an accurate model of morphology, complexity, and heterogeneity of human tumors [155, 156]. More promisingly, extensive work has been published in the last two decades to overcome the EPR hurdles, using mild hyperthermia and sonoporation to improve drug accumulation and penetration within the tumor mass [157-160]. We believe that the future prospects and success of tumor microenvironment targeted therapies rely on establishing strong validated models that truly represents tumors in cancer patients. Additionally, the use of combinatorial synergistic chemotherapeutics, that nanomedicine could offer, may provide a means to overcome tumor complexity, providing a more favourable realistic outcome.

Our review suggests that drug combination and time of therapy are the key factors that should be taken into consideration when designing combinatorial therapies for hypoxia, especially when translated to clinical trials. Previous pre-clinical studies have provided evidence that angiogenesis inhibition through TAM targeting can indeed inhibit tumor growth [161, 162]. Most importantly, TAM depletion which potentiates tumor hypoxia could be overcome if correctly combined with angiogenesis and/or hypoxia-targeted therapies [161, 162]. Although targeting TAMs and hypoxia individually has shown a great promise, we believe that developing combinatorial therapies exploiting both targets may ensure strong synergistic effects, and render solid tumors more susceptible to conventional cancer therapies. Such strategies can provide a sophisticated approach

to target multiple signaling pathways in tumorigenesis, and overcome off-target effects from positive loop feedbacks generated between TAMs, hypoxia and the tumor microenvironment. These studies provide evidence that simultaneously targeting TAMs and hypoxia is an interesting novel approach to exploit in cancer therapy.

Despite the apparent controversy and disappointing results discussed above, researchers must not forget the high number of newly approved drug delivery systems and ongoing clinical trials [152]. Therefore, we support aforementioned opinions that targeting the tumor microenvironment must be revised, and translation to clinic must be supported by adequate pre-clinical evaluation alongside standardized patients selection. Researchers need to focus not only on showing sophisticated nanomedicine with enhanced efficacy, but they should discover new techniques that can identify new tumor microenvironment biomarkers that could offer a personalised medicine for cancer patients. This can lead to the development of new targeted systems, and also aid in identifying tumors that will respond better to certain drug regimen. Efforts are in place to take nanomedicine closer to clinical translation by producing more reliable and positive treatment outcomes.

6. Conclusions

Significant progress has been made on deciphering the role of hypoxia and MACs infiltration in tumor malignancy. In the present work, we give an in-depth review on the active role of the tumor microenvironment in cancer survival and the development of drug resistance. TAMs have been considered as potential therapeutic targets for cancer therapy, since they are strongly linked to hypoxia and cancer progression. MACs have been envisioned as 'trojan horses', given their enhanced extravasation in hypoxic tumor cores. These features offer a great opportunity to design highly selective and sophisticated drug delivery systems that simultaneously target TAMs and tumor hypoxia. We believe that adopting this new approach is anticipated to overcome drug resistance and enhance the efficacy of chemotherapy in advanced solid and metastatic tumors. Finally, with validated tumor models and standardized patient pre-selection, and targeting hypoxia and TAMs, combinatory nanomedicine can become a more reliable therapeutic tool with more favourable clinical outcomes in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

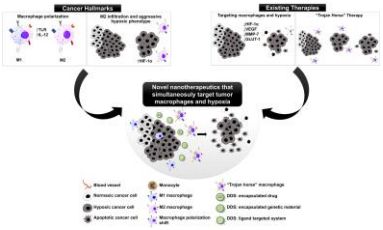
Fig 1. Main hallmarks of cancer. The cancer niche is a complex network of endothelial, stromal and malignant cells, comprised of evolutionary genomic features that enhance survival and thrive tumor cells to uncontrolled proliferation and metastasis. This enriched tumor microenvironment supports a shifted metabolism in cells, which allows a quick preadaptation and survival under nutrient and oxygen deprivation (hypoxia) which lead to therapeutic resistance.

Fig 2. The hypoxic tumor environment and its role in oncogenesis. Deprivation of oxygen in the tumor core has been linked to tumor development and poor prognosis. Upregulation of HIF-1 has shown to enhance the expression of many cancer markers related to: (i) tumor angiogenesis, (ii) invasion and metastasis, (iii) metabolism shift and (iv) infiltration of M2 macrophages in the (v) hypoxic regions of tumors. The futile cycle created by the positive feedback of negative prognosis biomarkers in cancer are responsible for tumor survival and resistance to therapy.

CCL17, Chemokine (C-C Motif) Ligand 17; **CCL22**, Chemokine (C-C Motif) Ligand 22; **COX-2**, cyclooxygenase-2; **CXCL12,** Chemokine (C-X-C Motif) Ligand 12; **CXCR4**, Chemokine (C-X-C Motif) Receptor 4; **EGF**, Epidermal growth factor; **FGF2**, fibroblast growth factor 2; **HIF-1**, Hypoxia inducible factor 1; GLUT-1, Glucose transporter 1; **MMP-7**, Matrix metalloproteinase 7; **MMP-9**, Matrix metalloproteinase 9; **MMP-12,** Matrix metalloproteinase 12; **PGK**, Phosphoglycerate Kinase 1; **TNF-α**, Tumor necrosis factor alpha; **VEGF**, Vascular endothelial growth factor

Fig 3. Nanoparticle-based therapeutics targeting TAMs and hypoxia. Drug delivery systems (DDS) have shown great promises to inhibit the effect of TAMs and hypoxia on cancer development. The main mechanisms that have been developed so far using nanoparticles to targets tumor microenvironment are: 1) inhibiting monocyte maturation, leading to a reduced accumulation of pro-tumor immune cells (M2) at the tumor site; 2) depleting the tumor from M2 macrophages; 3) shifting the polarization of M2 macrophages (pro-tumor state) to an anti-tumor M1 state; 4) downregulating HIF-1with specific inhibitors and ligands; and 5) using macrophages as trojan horses to deliver DDS to the hypoxic regions of the tumor.

CCL2, Chemokine (C-C Motif) Ligand 2; **DDS**, Drug delivery system; **HIF-1**, Hypoxia inducible factor 1; **GLUT-1**, Glucose transporter 1; **IL-12,** Interleukin 12; **M-CSF**, Macrophage colony stimulating factor; **MMP-7**, Matrix metalloproteinase 7; **MMP-9,** Matrix metalloproteinase 9; **TLR**, Toll-like receptor; **VEGF**, Vascular endothelial growth factor



Graphical abstract