Nanomedicine-mediated drug targeting of cancer stem cells

Deepika Singh, Aliva Prity Minz and Sanjeeb Kumar Sahoo

Institute of Life Sciences, Nalco Square, Chandrasekharapur, Bhubaneswar, India

Tumors are heterogeneous and contain a small population of cells that has a crucial role in tumor progression, metastasis, drug resistance, and relapse as a result of their self-renewal, proliferation, and differentiation properties. These cells are known as cancer stem cells (CSCs) and accumulating evidence suggests that they show significant resistance to conventional chemotherapy. Thus, various antitumor strategies have been developed to eliminate therapeutic-resistant CSCs by targeting the molecular differences between CSCs and bulk cancer cells. Here, we highlight the use of nanomedicine-mediated dual drug delivery to target CSCs and bulk cancer cells simultaneously. We also summarize current prospects and challenges associated with this therapy.

Introduction

Various studies have delineated subpopulations of cells within tumors that differ in terms of their self-renewal capacity, long-term proliferation potential, capacity to seed new tumors when implanted in an appropriate host, and cancer recurrence potential; such cells are known as CSCs or tumor-initiating cells (TICs) [1,2]. In 1987, Bonnet et al. were the first to provide evidence for the existence of CSCs, documenting that the driving force for human acute myeloid leukemia was a small fraction of CD34+/CD38− leukemic stem cells that were able to transfer the disease to NOD/SCID mice [3]. Since their initial discovery, evidence has accumulated for the presence of CSCs in most solid carcinomas. With advances in new technologies, several features of CSCs have been revealed, including their recurrence, metastasis, multidrug resistance, dormancy, and survival under hypoxic conditions. As a result of their tumor-initiating ability and resistance to conventional chemotherapy and radiotherapy, CSCs have emerged as targets for cancer therapy in recent years. Conventional treatment with chemotherapy and radiotherapy can kill bulk tumor cells but fail to result in long-lasting clinical outcomes, because they are ineffective against CSCs. Thus, following conventional treatment, the presence of CSCs leads to tumor relapse [4].

Studies over the past few decades have revealed that tumors are heterogeneous tissues comprising phenotypically distinct cell populations. CSCs display plasticity via their reversible transition between the stem and non-stem cell states. Therefore, depletion of CSCs alone might not be sufficient for complete tumor regression because it is likely that the differentiated tumor cells are capable of becoming CSCs and, thus, sustaining tumor growth [5,6]. To develop successful therapeutics against CSCs, a more complete understanding of the characteristics of CSCs and the appropriate application of modern technologies for the delivery of different therapeutics to block their proliferation remain major challenges. In recent years, the nanotechnology-based delivery of different therapeutics (i.e., nanomedicines) has shown potential for the treatment of CSCs compared with conventional therapeutics by overcoming some of the limitations of the latter, such as poor water solubility, poor pharmacokinetics, and poor stability of CSC-specific agents; these improvements enhance the penetration of the drugs into the CSC niche, leading to reduced chances of tumor relapse [7]. Here, we summarize the role of CSCs in tumorigenesis and the potential sites for therapeutic targeting. We also highlight different nanomedicine drug delivery approaches, including combinational strategies resulting in synergistic effects that completely eradicate the tumor, which could be translated into the clinic in the near future.
Cancer stem cells and tumorigenesis

Fig. 1 highlights the main characteristics of CSCs that account for the failure of chemotherapy against these cells, and we discuss here some of the crucial properties of CSCs that are responsible for tumorigenesis. A decade ago, it was reported that aging and environmental signals lead to the generation of an epigenetically disrupted progenitor cell pool by affecting certain genes before the emergence of oncogenic mutations leading to cancer [8]. As key players in tumorigenesis, epigenetic mediators have a major role in the emergence and maintenance of CSCs, metastasis, and tumor progression. Further work also revealed that CSCs are either primarily glycolytic or preferentially rely upon oxidative phosphorylation in a tumor type-dependent manner and, moreover, that cellular metabolism controls their stemness properties [9]. In addition to glucose, CSCs might also rely upon mitochondrial fatty acid oxidation for NADPH and ATP production [10]. Studies confirmed that the epithelial-to-mesenchymal transition (EMT) in epithelial cells also has a lead role in metastasis, resulting in the disturbance of epithelial cell homeostasis and procurement of a migratory mesenchymal phenotype, which results in the migratory properties of CSCs [11]. CSCs have also been shown to express multifunctional efflux transporters, such as ATP-binding cassette (ABC) transporters, as protection against damage and death. These ABC transporters are responsible for shielding CSCs from adverse effects of chemotherapeutic insults [12]. An additional crucial factor in the chemotherapeutic resistance of CSCs is their quiescence state, which also has a significant role in their self-renewal [13]. Recently, it was found that CSCs show immune escape via different biological mechanisms, such as the downregulation of major histocompatibility complex (MHC) class I and class II molecules, inadequate antigen presentation, and release of immunosuppressive factors that prevent their recognition or destruction by the host immune system [14]. It was also revealed that CSCs are resistant to DNA-damaging agents because they have a highly efficient DNA damage response system for processing DNA damage more efficiently compared with bulk cancer cells [15].

Advantages of nanomedicines in targeting cancer stem cells

Nanotechnology shows immense scope for the development of a drug delivery system that can address the urgent need for drugs with improved therapeutic efficacy against CSCs. Nanoparticle (NP)-based carriers (nanocarriers), such as micelles, polymeric NPs, liposomes, and dendrimers, are becoming the preferred choice of drug delivery system. Although many anticancer agents have been proposed, most show limitations when translated to clinical studies, including off-target effects, a hydrophobic nature, low pharmacokinetics, inconsistent stability, and unfavorable biodistributions. Nanotechnology paves a way to overcome these limitations and addresses the urgent need to improve the efficacy of cancer diagnosis and therapies [16]. Over the past few years, researchers have focused on designing a single nanoformulation that carries dual drugs (one specific against CSCs and a second against bulk tumor cells) for delivery to the target site via active or passive targeting, with drug release achieved either passively or via a triggered mechanism (Fig. 2).

Passive targeting exploits the leaky vasculature and poor lymphatic drainage of the tumor niche that results from rapid and defective angiogenesis; this enables macromolecules to accumulate and become trapped within the tumor bed. However, because this form of targeting solely depends on the diffusion of the drug, it is affected by the fact that not all tumor vessels share similar permeability and that some drugs might not diffuse as efficiently as others. These limitations can be overcome by active targeting, whereby the targeting moiety is conjugated to a...
nanoformulation that can specifically bind the targeting receptor or the antigen on the cell surface. Such an approach is used to eradicate CSCs situated deep inside the tumor tissue because of the endocytic delivery mode and increased cellular uptake of the drug [17]. In addition, nanoformulations can act as high-capacity drug carriers and display enhanced bioavailability and activity. Studies have also confirmed that nanomedicines could evade efflux by ABC drug efflux pumps because their mode of entry is receptor-mediated, energy-dependent endocytosis. In addition, because nanomedicines accumulate at the tumor site as a result of impaired vasculature, this prevents any adverse effects on normal stem cells. Thus, rationally designed nanomedicines can open doors for CSC-specific therapies by penetrating the CSC niche [4].

**Targeting cancer stem cells: a hallmark in cancer management**

Researchers are targeting CSCs because of their major role in tumor progression, tumor maintenance, development of multidrug resistance, and metastasis. Targeting CSCs is challenging because various targeting strategies are required for the simultaneous eradication of CSCs and non-CSCs in the tumor tissue. Given that CSCs are molecularly distinct from non-CSCs, these molecular differences can be exploited for targeting the former [18] (Fig. 3). Certain signaling pathways, such as Wnt, Hedgehog, Notch, and PI3K/AKT, are particularly important because they are deregulated in cancers, promoting the survival and self-renewal of CSCs [19].

**Targeting the quiescence of CSCs**

One reason for the therapeutic resistance of CSCs might be their quiescent state, which resembles the nondividing state of a cell. Conventional treatments, such as chemotherapy and radiotherapy, preferentially target actively dividing cells. Thus, quiescence aids the escape of CSCs from standard treatment and confers therapy resistance. Certain proteins, such as Fbw7 and Bcl-X_L, are responsible for CSC quiescence. Fbw7 is a member of the F-box family of proteins and interacts and mediates the ubiquitination of c-Myc, cyclin E, Notch, c-Jun, and Mcl-1, degradation of which is essential for the maintenance of quiescence. Endogenous cyclin-dependent kinase inhibitors and Fbw7 contribute to cell cycle delay or arrest in CSCs. Thus, Fbw7 acts as a tumor suppressor, inactivation of which promotes the growth of quiescent CSCs in the tumor niche [20,21]. Thus, therapeutic strategies combining the inhibition of Fbw7 with conventional anticancer drugs to prevent CSCs entering the G0 quiescent phase could be useful for overcoming the chemotherapeutic resistance of CSCs [22]. Zeuner et al. showed that quiescent and/or slow-proliferating lung CSCs depend strongly on Bcl-X_L for their survival and claimed that inhibition of Bcl-X_L by ABT-737 (a Bcl-2/Bcl-X_L inhibitor) could be a potential therapeutic avenue for the treatment of non-small-cell lung cancer [23]. Cytokines, such as G-CSF and IFN-α, as well as arsenic trioxide, can efficiently activate dormant hematopoietic stem cells and leukemic stem cells, making them more susceptible to chemotherapy [24].

**Targeting by induction of CSC apoptosis**

Apoptosis is a biological phenomenon that removes damaged or abnormal cells. However, specific mutations at the gene level help tumor cells to evade apoptosis, leading to malignancies. Pathways that govern the activation of prosurvival signaling and inactivation of apoptotic signaling pathways could be crucial means by which CSCs confer drug resistance and avoid apoptosis. Some tumors express high levels of antiapoptotic Bcl-2 family proteins, including Bcl-2, Bcl-X_L, and Mcl-1, contributing to chemotherapeutic resistance by affecting the apoptotic threshold of neoplastic cells [25]. In this context, Ma et al. developed berbamine-loaded liposomes and evaluated the efficacy of this formulation against breast CSCs in vitro and in vivo in a xenograft model. The authors reported that berbamine-loaded liposomes activated Bax (a proapoptotic protein) and inhibited Bcl-2 (an antiapoptotic protein), which further reduced the tumor volume in vivo [26]. In similar
FIGURE 3

Possible different molecular pathway targets of cancer stem cells (CSCs). (a) Signal transduction pathways: Wnt, Notch, and Hh are responsible for maintaining the properties of CSCs. Possible potential targets within these pathways include β-catenin, γ-secretase, and Smo, as well as inhibiting ligand–receptor interactions. (b) Apoptosis: increasing the accumulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) activates Caspase (Casp) 8, whereas inhibition of inhibitors of apoptosis proteins (IAPs) negatively activates Caspase 9. Activated Caspase 8 and 9 further activate Caspase 3, leading to apoptosis induction. (c) Quiescence: Fbw7 negatively regulates cell cycle progression via the ubiquitin-mediated degradation of c-Myc. Inhibiting Fbw7 can lead to the

study, Zhang et al. developed daunorubicin with quinacrine-conjugated liposomes that activated Bax, leading to breast CSC death [27].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an apoptosis-inducing ligand that activates Caspase-8 and -10, downstream apoptotic ‘executioners’ that autoactivate by proteolysis. Activated caspase-8 and -10 then cleave caspase-3, resulting in cell death. Several TRAIL receptor agonists are currently in Phase 1 and 2 trails. Yin et al. described the effectiveness of a cisplatin and TRAIL co-treatment in reducing triple-negative breast CSCs via increasing apoptosis and inhibiting the Wnt signaling pathway [28].

Recently, various natural polyphenolic compounds have been shown to induce apoptosis in breast CSCs. For example, Pandey et al. reported that resveratrol induced apoptosis in breast CSCs by modulating the fatty acid synthetase survival pathway [29]. Hence, targeting CSCs by induction of apoptosis offers a possible approach to cancer eradication, as supported by preclinical studies.

**Targeting by induction of CSC differentiation**

Conventional anticancer therapies are successful in managing differentiated cancerous cells but fail against CSCs because of their undifferentiated state. Thus, the induction of CSC differentiation could be a novel approach to killing CSCs. Researchers have found that retinoic acids and drugs targeting epigenetic changes are effective against CSC differentiation [30]. Recently, Campos et al. showed that stem-like glioma cells (SGLC) differentiated when treated with all-trans retinoic acid (ATRA). The in vitro differentiation sensitized these cancer stem-like cells to chemotherapy and inhibited the secretion of angiogenic cytokines and metastasis. Further ex vivo differentiation reduced the tumorigenicity [31]. Similarly, Nguyen et al. also reported that ATRA induced the differentiation of the tumorosphere in 3D culture, inhibiting its growth by arresting the cell cycle in vitro. In vivo studies showed that ATRA significantly inhibited gastric tumor progression by further downregulating the stem cell marker CD44 and ALDH activity [32]. Currently, much attention is being given to developing a nanoformulation that encapsulates a differentiating agent with a conventional drug to differentiate CSCs into bulk cancer cells that then become susceptible to conventional chemotherapy. For example, Sun et al. designed PEG-b-PLA NPs encapsulating ATRA targeting CSCs and doxorubicin as a traditional chemotherapy drug and showed the synergistic inhibition of CSCs both in vitro and in vivo against breast cancer [33].

**Inducing differentiation by epigenetic regulation**

Epigenetic regulation also governs the differentiation and self-renewal properties of CSCs; thus, modulating the epigenetic structure using chromatic modifiers could be an encouraging strategy to control CSC fate [30]. Histone deacetylase (HDAC) expression is often altered in most malignancies. Histone acetyltransferase (HAT)/HDAC maintains the balance between histone acetylation and deacetylation. Hence, epdrugs that modulate the histone code could be potential anti-CSC therapy agents. Salvador et al. reported the ability of the HDAC inhibitor (HDACi) abexinostat to induce CSC differentiation and reduce the CSC population in patient-derived xenograft models with low expression of the biomarker RNA Xist [34]. Hence, targeting CSCs by means of differentiation shows therapeutic potential.

**Inducing the mesenchymal-to-epithelial transition**

The EMT and mesenchymal-to-epithelial transition (MET) are crucial events for CSC metastasis. EMT is a phenomenon whereby epithelial cells acquire fibroblast-like properties, lose their cell-cell adhesion, and increase their motility, facilitating the escape of tumor cells from primary tumors. Hence, EMT confers mesenchymal traits on both normal and neoplastic epithelial cells. Activation of EMT enables both these classes of cells to acquire stemness [35,36]. CSCs of different carcinomas, including breast, and head and neck squamous cell carcinomas, show mesenchymal attributes, indicating that these cells have passed through the EMT at least partially [37,38]. Thus, the EMT is relevant to the maintenance and acquisition of stem cell-like characteristics and is sufficient to endow stem cell properties to differentiated normal and cancer cells [39,40]. At the site of metastasis, the disseminated mesenchymal tumor cells undergo the reverse transition, the MET [35]. This link between EMT, MET, and CSCs presents attractive opportunities for drug development via agents targeting specifically more mesenchymal carcinoma cells, rather than their epithelial counterparts, to eliminate CSCs.

One way to target mesenchymal CSCs is to develop therapeutics that are specific and cytotoxic towards these cells [2]. An alternative novel strategy has been described in which CSCs can be induced to exit their mesenchymal tumor-initiating state and acquire an epithelial nonstem-like state. This induced differentiation can make cells more vulnerable to conventional cytotoxic drugs. Umbrecht et al. recently showed that lapatinib is capable of inducing MET in head and neck squamous cell carcinoma, as depicted by the downregulation of vimentin and upregulation of E-cadherin [41]. Pattabiraman et al. described the role of cAMP and its downstream target, protein kinase A (PKA), in regulating MET. cAMP, a secondary messenger, is an activator of PKA, and activation of the latter either by cAMP or another agent is required for MET. Thus, by increasing the level of cAMP via drugs such as forskolin or a cAMP analog, such as 8-chlorophenylthiocAMP, will further activate PKA, leading to the differentiation of mesenchymal CSCs into epithelial cells, rendering them more susceptible to conventional therapeutic drugs [42]. Until recently, nanomedicines served as a carrier for existing therapeutic agents; however, Liu et al. provided evidence that the metallofullerener nanomaterial Gd@C82(OH)22 blocked the EMT, and resulting in the differentiation of mesenchymal breast CSCs into their epithelial phenotypes by blocking TGF-β signaling [43].

**Targeting by blocking cell signaling pathways**

Cell signaling can act via the direct interaction of a cell with its neighbor or by the action of signaling molecules, such as
epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), and insulin. CSCs utilize most of the same signaling pathways found in normal stem cells during embryonic development.

**Targeting embryonic signaling pathways**

It is accepted that tumors often progress because of deregulated self-renewal and aberrant signaling pathways. Embryonic signaling pathways, especially Wnt, Notch, and Hedgehog (Hh), are important for self-renewal and differentiation in CSCs. Thus, these signaling pathways are important because they could be potent targets for novel therapeutics. Recently, several therapeutics that target these signaling pathways have reached various clinical trial phases [17].

For an example, Hh signaling has a significant role in controlling the regulation of tissue polarity, pattern maintenance, and cell fate determination. The interaction between Hh and its receptor patched (Ptch) activates Hh signaling. In the presence of Hh, Ptch is inhibited, allowing smoothened (sma) activation, which in turn activates the Gli family of transcription factors, which induces several gene transcripts that are responsible for cell survival and EMT [44]. Several specific Hh inhibitors have recently been studied, such as cyclopamine (steroid alkaloid) and vismodegib (a GDC-0449-cyclopamine derivative), which are claimed to reduce cell viability and induce apoptosis in CSCs. Recently, Yoon et al. showed that GDC-0449, an inhibitor of Smo, decreased the formation of CD44+ tumorosphere and further increased the sensitivity to different antineoplastic drugs, including 5-fluorouracil and cisplatin, substantially reducing the cell viability compared with individual neoplastic drugs [45]. In another study, Verma et al. found that an antimalarial compound, anthothecol, isolated from *Khaya anthotheca*, and conjugated with poly(lactic-co-glycolic acid) (PLGA) NPs, inhibited the self-renewal property of CSCs by inhibiting the Hh signaling pathway in a dose-dependent manner [46].

Notch signaling is important because it is activated via direct cell–cell contact. Once the binding of ligand with its receptor occurs, the notch receptor exposes the site for cleavage by γ-secretase [47], releasing active notch intracellular domain (NICD), which moves to the nucleus and allows transcription of notch target genes, such as Myc, HES, or p21. It was recently highlighted that notch signaling has a role in drug resistance and the downregulation of notch signaling increased drug sensitivity, inhibited tumor regrowth, and reduced metastasis in CSCs [48]. Therapeutic agents targeting γ-secretase, Delta-like ligand (DLL), and notch receptors can be effective in reducing cancer progression. Seveno et al. reported that the γ-secretase inhibitor (GSIXII: Z-ILE-ILEU-ALDEHYDE) alone prevented mammosome formation, whereas GSIXII in combination with ABT-737 (an inhibitor of antiapoptotic proteins) led to an enhanced synergistic apoptosis response as depicted by *ex vivo* assays [49]. Recently, Liu et al. fabricated paclitaxel-loaded polymeric NPs conjugated with GD1b peptide (H2N-GRCTNHFNIYCFPDP-COH2), which specifically targeted the angiogenic marker DLL4. This nanomedicine exhibited profound tumor neovascularization-targeting capabilities *in vivo* [50]. Another study by Yen et al., in pancreatic xenograft tumor models, showed that an anti-human DLL4 antibody (OMP-21M18) in combination with gemcitabine had an additive effect by targeting DLL4 [51]. Recently, this research group also developed an anti-Notch2/3 antibody OMP-59R5 (tarectumab) that blocked notch functions in combination with gemcitabine. Furthermore, the antitumor effect was enhanced when nab-paclitaxel was added to this combination, as validated by xenograft experiments [52].

The Wnt/β-catenin signaling pathway is one of the most deregulated pathways in many cancers and is considered a key factor in the self-renewal and maintenance of CSCs. Via several downstream processes, the signal is transferred to β-catenin, which translocates to the nucleus and activates the transcription of Wnt target genes [53]. Targeting the Wnt/β-catenin pathway using therapeutic agents can inhibit malignant conversion, tumour aggressiveness, and improve clinical outcome [48]. Researchers are attempting to inhibit the Wnt signaling pathways by developing novel inhibitors, such as ICG001 and XAV-939 (developed by Prismbiolab and the National Cancer Institute, respectively), targeting β-catenin. Curcumin, a phytochemical extracted from *Curcuma longa*, is gaining much attention for targeting several signaling pathways in CSCs. In Wnt signaling, curcumin specifically decreases the expression of β-catenin [54]. An antimicrobial drug, salinomycin, has also been reported to have an anti-CSC effect by inducing DNA damage and inhibiting the Wnt signaling pathway. A study showed that conjugating paclitaxel and salinomycin targeted to breast cancer and CSCs, respectively, with CD44 antibody conjugated single-walled carbon nanotubes, had an enhanced therapeutic effect both *in vitro* and *in vivo* compared with individual drug-conjugated nanocarriers or free drug. Hence, this nanomedicine holds great promise as an effective breast cancer treatment [55].

**Crosstalk among signaling pathways**

Crosstalk refers to the transfer of signals among different signaling pathways. This communication between Notch, Hh, Wnt, Akt, and other signaling pathways has been reported in various cell types. Crosstalk among signaling pathways further adds to the complexity of CSCs. CSCs can offer certain opportunities for inhibiting multiple cascades by targeting a single pathway [44]. An example of this kind of targeting was provided by Kwon et al., who showed that an inhibitor of the Notch pathway (PF-03084014) was also capable of inhibiting the Wnt pathway by post-transcriptionally decreasing β-catenin levels [56].

A connection between notch signaling and mammalian target of rapamycin (mTOR) suggests that cells that have activated mTOR also express elevated levels of NICD and Hex1, which are the key transcription factor in Notch signaling [57]. Downregulation of notch signaling also mediates the inhibition mTOR, Akt, and NF-κB signaling [58]. Although rapamycin and its analogs, which are allosteric inhibitors of mTORC1, are potent immunosuppressors, they also demonstrate low anticancer activity in patients. This might be because they are capable of inhibiting only one of the several downstream targets of PI3K, while leaving Akt unaffected. By contrast, dual inhibitors, such as VS-5584, which inhibit both PI3K and mTORC1/2 were found to be potent anticancer agents in both *in vitro* and *in vivo* experiments against CSCs [59]. This suggests that dual inhibitors of the PI3K/mTOR signaling pathway can seed novel therapeutic approaches against CSCs. The combined inhibition of sonic Hh and mTOR with cyclopamine and rapamycin, respectively, along with gemcitabine (used as a
standard therapy) diminished pancreatic CSCs both in vitro and in vivo, whereas neither cyclophamine nor rapamycin alone was effective in eliminating the CSC population [60]. Thus, an improved understanding of crosstalk networks is likely to support the design of effective anticancer therapies.

Concluding remarks and future directions

Our knowledge and understanding of CSCs have both improved over the past few decades, and many new targets of CSCs have been discovered. Here, we have summarized the importance of CSC elimination for complete eradication of a tumor. The novel therapeutic strategies described herein show promise for targeting CSCs directly along with other non-CSCs by interfering with signaling cascades or the differentiation of the cells. We have also focused on nanomedicinal approaches that can specifically deliver the therapeutic in a targeted manner. Although significant achievements have been made in this area, to ensure the clinical success of these nanomedicines in the form of ‘magic bullets’, continued research efforts are required to attain a comprehensive understanding of the properties of CSCs to uncover additional novel targets and to develop more-efficient nanoformulations as delivery systems. Another challenge associated with nanomedicine is the change in the drug toxicity profile after loading on a nanovehicle. Nanomedicines can also show independent toxicity effects, which are an additional complicating factor and, therefore, must also be an area of future investigation. We speculate that, in the near future, additional novel targets in CSCs will be uncovered and the benefits of the target-based nanomedicine approach described above will provide additional benefits for patients with cancer.

Acknowledgments

D.S. would like to acknowledge the Institute of Life Sciences for providing an institutional Junior Research Fellowship; A.P.M. would like to acknowledge the Council for Scientific and Industrial Research for providing a Junior Research Fellowship.

References

42 Pattabiraman, D.R. et al. (2016) Activation of PKA leads to mesenchymal-to-epithelial transition and loss of tumor-initiating ability. Science 351, 63680

59 Kolev, V.N. et al. (2015) PI3K/mTOR dual inhibitor VS-5584 preferentially targets cancer stem cells. Cancer Res. 75, 446–455