Molecular Pathways: Involving Microenvironment Damage Responses in Cancer Therapy Resistance

Yu Sun and Peter S. Nelson

Abstract
The armamentarium of therapeutics used to treat cancer patients relies heavily on ionizing radiation and chemotherapeutic drugs that severely damage DNA. Tumor cells' responses to such treatments are heavily influenced by their environment: Physical contacts with structural elements such as the extracellular matrix, associations with resident and transitory benign cells such as fibroblasts and leukocytes, and interactions with numerous soluble endocrine and paracrine-acting factors all modulate tumor-cell behavior. Of importance, this complex tumor microenvironment is not static and dynamically responds to a variety of stimuli. Here, we describe emerging data indicating that genotoxic cancer treatments activate highly conserved damage response programs in benign constituents of the tumor microenvironment. These damage signals, transmitted via master regulators such as NF-xB, culminate in a powerful and diverse secretory program that generates a proangiogenic, proinflammatory microenvironment. Constituents of this program include interleukin (IL)-6, IL-8, hepatocyte growth factor, amphiregulin, matrix metalloproteinases, and other factors that have been shown to promote adverse tumor-cell phenotypes, such as enhanced resistance to treatment and rapid tumor repopulation. A detailed understanding of these survival signals induced in the context of genotoxic stress provides a platform for developing combinatorial treatment strategies that take into account malignant cells, the tumor microenvironment, and the dynamics exerted by the treatment itself. Clin Cancer Res; 18(15); 4019–25. ©2012 AACR.

Background
Since the advent of modern cancer therapeutics that involve the administration of drugs and ionizing radiation to eradicate neoplastic cells, both de novo and acquired resistance have been recognized as major barriers to cures. Most cancer-directed therapies fall broadly into 3 classes that exploit differential vulnerabilities in malignant tumors relative to benign tissue counterparts. The most commonly deployed therapies inflict substantial damage to nuclear DNA or cell division machinery, resulting in genotoxic catastrophe or the engagement of damage response mechanisms that halt cell proliferation. However, the lack of specificity of these interventions limits doses to avoid collateral damage to normal tissues. A second category of cancer therapeutics has emerged through a detailed understanding of oncogenic pathways that direct targeted inhibition of key drivers such as kinases, growth factors, and growth factor receptors. A third approach to treat cancer exploits mounting information implicating the important contribution of the microenvironments within which tumor cells develop, proliferate, and (in the case of metastasis) colonize and occupy distant sites. Such strategies include inhibiting new vasculature and augmenting immune system responses.

Each of the above categories of cancer treatments includes agents that are capable of markedly suppressing tumor growth, but each also suffers from failures due to the engagement or selection of resistance programs. Tumor–cell–autonomous or intrinsic resistance mechanisms such as the activation of multidrug resistance efflux pumps, activation of bypass signaling pathways, and secondary mutations in drug targets are well established, and designs of therapeutics have iteratively evolved to exploit these molecular alterations. Less well studied are factors that contribute to cell–nonautonomous or extrinsic mediators of therapy resistance, such as those provided by nonmalignant cells and structural constituents of the tumor microenvironment. Recent work has defined niches within tissues and organs that offer sanctuary to tumors and activate therapy resistance programs. In several notable instances, efforts to exploit these tumor–host dynamics have led to successful clinical translation to affect patient survival. Here we discuss mechanisms by which tumor and host interactions in the microenvironment influence treatment resistance, with an emphasis on reactions and responses induced by the cancer therapeutics themselves that have the potential to attenuate treatment lethality and paradoxically promote tumor cell survival.
Therapy Resistance

Tumor microenvironment

Neoplasms arise and grow in complex and dynamic ecosystems. For most types of solid tumors, the microenvironment is comprised of numerous resident benign cell types derived from distinct developmental lineages, as well as nonresident cell types that may be transient or may persist to become permanent components of an evolving interactive biowork. A structural framework provided by insoluble matrix proteins and gradients of diffusible growth factors, hormones, oxygen, reactive oxygen species, and nutrients adds to the complexity.

Of importance, many facets of the tumor microenvironment are capable of profoundly influencing the behavior of preneoplastic and overtly malignant cells. In contrast with fibroblasts derived from benign tissues, cancer-associated fibroblasts can augment the growth of preneoplastic cells and influence invasive tumor behavior in a number of organs, including the prostate, breast, and stomach (1–3). Likewise, inflammatory cell components such as B cells, T cells, and macrophages can promote adverse cancer phenotypes within the skin, breast, and other tissues (4, 5). The existence of a temporally dynamic microenvironment is evident in studies such as those showing that a normal young liver microenvironment is tumor suppressive, whereas a normal aged liver microenvironment is permissive for tumor establishment and progression (6). Similarly, detailed studies of tumor hypoxia, pH, angiogenesis, and rigidity have clearly shown that these and other attributes of the microenvironment can produce major changes in tumor phenotypes.

Although recent findings emphasize the importance of studying tumor characteristics, such as proliferation and invasion, in the context of the multidimensional influences exerted by the tumor microenvironment, there is less information concerning the roles played by the microenvironment in resistance to cancer therapeutics. However, it is well recognized that ex vivo assays of chemotherapeutics poorly recapitulate in vivo effectiveness (7). In part, these differences clearly reflect drug delivery issues related to vascular access, interstitial pressures, and metabolism (8). However, other elements of the tumor microenvironment can affect tumor phenotypes to augment drug resistance. Distinct microenvironments can provide niches that contribute substantially to tumor cell survival and eventual relapse and therapy failure. A few examples serve to illustrate the variety of ways in which the context provided by the tumor microenvironment can influence tumor resistance to therapeutics (for reviews, see refs. 8–10), as described below.

Therapy resistance mediated by soluble factors. As with chronic myelogenous leukemia (CML), Philadelphia chromosome-positive acute lymphocytic leukemia (Ph+ ALL) is driven by the BCR-ABL fusion protein and is sensitive to the Abl tyrosine kinase inhibitor imatinib (11). In a series of experiments reported by Williams and colleagues (12), a mouse model of Ph+ ALL was developed that exhibited resistance to imatinib, although Abl kinase activity was inhibited by drug treatment. However, tumor cells isolated from this model were still sensitive to imatinib in vitro, supporting the hypothesis that components of the host microenvironment (in this case, the hematopoietic microenvironment) promote resistance. Through further experimentation, host cytokines, such as interleukin (IL)-7, were determined to promote growth despite imatinib treatment (12). Other studies implicated galectin 3 induction in CML by the bone marrow microenvironment as a contributing factor to drug resistance and long-term lodgment of leukemic cells in the bone marrow niche (13). Further evidence for this mechanism of therapy resistance (described below) points to the role(s) of cancer therapeutics themselves in promoting the production of microenvironment-derived soluble factors.

Therapy resistance mediated by physical barriers. Cancers that arise from pancreatic duct cells are highly lethal and show quite limited responses to radiation or chemotherapy. However, cell lines and xenografted tumors derived from pancreatic cancers do exhibit responses to many chemotherapeutic drugs, such as gemcitabine, an agent used commonly to treat pancreatic cancer patients with modest efficacy (14). In a series of insightful studies using the KDM genetically engineered model of pancreatic cancer, Olive and colleagues (15) found a marked difference between tumor cells grafted into subcutaneous sites and cancers arising within the environment of the in situ pancreas in terms of responses to chemotherapy. They also found that major contributors to these differential tumor responses were limited vascularity and poor perfusion, which constrained drug penetration within the pancreas. The efficacy of chemotherapy was substantially enhanced through the use of IPI-926, a sonic hedgehog pathway inhibitor that depleted tumor-associated stromal tissue, increased tumor vascularity, increased intratumoral chemotherapy concentrations, and consequently inhibited tumor growth (15).

Therapy resistance influenced by cell adhesion. Physical interactions between multiple-myeloma cells and structural constituents of the bone marrow have been shown to profoundly influence de novo and acquired resistance to chemotherapy (16). Mechanisms that contribute to adhesion-mediated resistance include tumor-cell binding—via integrins and other components—to ligands on stromal cells and extracellular matrix such as fibronectin, collagens, and laminins. Consequent therapy resistance occurs through several pathways, including redistribution of the antiapoptotic proteins CASP8 and FADD-like apoptosis regulator from the cytoplasm to cell membranes, induced proapoptotic degradation of the proapoptotic protein BCL2-interacting mediator of cell death, and transient posttranslational upregulation of the cyclin-dependent kinase inhibitor p27 (17, 18). Of importance, drug sensitivity can be augmented by agents that disrupt adhesion. In preclinical studies, a blocking antibody to α4 integrin reduced tumor burden and increased overall survival in a mouse model of multiple myeloma, and dramatically augmented myeloma responses when used in conjunction with melphalan, a
drug in common clinical use for the treatment of multiple myeloma (19). Information about the key relationships between myeloma cells and the bone marrow microenvironment led to a series of rationally designed clinical trials that cotargeted tumor and microenvironment interactions. Lenalidomide, an agent that among other effects decreases tumor cell binding to bone marrow components, and bortezomib, a proteasome inhibitor that among other effects downregulates adhesion molecules on both tumor cells and bone marrow stroma, were both shown to substantially improve overall survival (20), and these agents are now part of the routine clinical management of patients with multiple myeloma.

Microenvironment reactions to cancer-directed therapeutics

It is important to consider that the effects of most cancer-directed therapeutics are not entirely restricted to neoplastic cells, and that such therapeutics can also interact with—and alter—benign cells in local and distant host microenvironments. The potential for such effects is particularly relevant for nonspecific treatments involving ionizing radiation and genotoxic drugs. Highly conserved damage and stress-response programs have evolved to prevent the propagation of oncogenic genetic damage to progeny by temporarily arresting cell growth for DNA repair, or irreversibly arresting growth through senescence or apoptosis.

DNA damage response. The DNA damage response (DDR) is a complex and coordinated process that occurs following a breach in the integrity of DNA (21). The DDR likely evolved to protect the host from cells that sustain irreversible genomic damage resulting from exposure to exogenous and endogenous genotoxins. The DDR culminates in the elimination of cells whose damage cannot be repaired. Common routine environmental insults and byproducts of cellular metabolism produce in excess of 1 million individual DNA lesions per cell per day (22). To deal with this assault, repair mechanisms are in continual operation, and the rate of repair is sufficient to manage the rate of damage. However, exposure to genotoxic cancer therapeutics produces damage that far exceeds the capacity of the repair process to maintain DNA integrity. Alkylating agents produce DNA interstrand cross-links, which promote DNA double-strand breaks. Topoisomerase inhibitors produce several effects, including the generation of interstrand cross-links, the creation of free radicals, and the stabilization of DNA with consequent inhibition of proper DNA replication and a consequent damage response signal. Platinum drugs induce DNA adducts and double-strand breaks, and the antibiotic bleomycin induces direct double-strand breaks. These and other chemotherapeutics engage the DDR to initiate fail-safe programs that result in permanent growth arrest (senescence) or the execution of cell death (apoptosis).

The DDR is enacted by the Mre11-Rad50-Nbs1 mediator complex, which denotes specific sites of damage, followed by a second phase that propagates the recognition signal to ultimately influence repair and cellular phenotypic responses. The DDR progresses through a signaling cascade that includes ATR and ATM (23). In the context of double-strand breaks resulting from chemotherapy, ATM autophosphorylates at multiple sites, self-activates, and instigates reactions that assemble checkpoint proteins such as p53BP1 and BRCA1 at the break site to promote damage repair (24–26). Concurrently, ATM activates CHK2, leading to the stabilization and accumulation of p53, a pivotal mediator of either pause and repair or permanent growth arrest and cell death. Although tumor cells commonly inactivate key components of the DDR program, benign cells of the tumor microenvironment are fully capable of producing robust responses to genotoxic stress. It has recently become apparent that in addition to the cell-autonomous components of the DDR that influence the damaged cell itself, the DDR also promotes a cell-nonautonomous program of secreted factors that are capable of affecting numerous cell types comprising the tumor microenvironment, including those tumor cells that have survived the first salvo of chemotherapy and radiotherapy.

DNA damage secretory program. The secretory phenotype of damaged cells was first reported in the context of cellular senescence, a state of permanent growth arrest. Cellular senescence, as described by Hayflick and Moorhead (27) in the context of replicative exhaustion, is associated with characteristic morphologic features encompassing enlarged flattened cell bodies with increased cytoplasmic granularity. Although their growth has been arrested, senescent cells remain viable and metabolically active (28). The mechanism behind replicative exhaustion involves the progressive erosion of telomeres after many replication cycles, with the consequent induction of a DDR-like response culminating in the induction of the CDK inhibitors p21 and p16 and permanent growth arrest (29). Investigators have identified several other inducers of senescence, including oxidative stress and reactive oxygen species, activation of specific oncogenes such as RAS and BRAF (30, 31), and profound levels of DNA damage, such as those encountered in the context of chemotherapy and radiotherapy.

Detailed studies of senescent cells revealed that this state is accompanied by the production and secretion of a remarkable spectrum of cytokines, growth factors, and proteases, many of which have been shown to play roles in promoting tumor growth and invasion (32–34). Collectively, these secreted factors have been termed a senescence-associated secretory phenotype (35) or senescence-messaging secretome (36). However, it appears that a full senescence phenotype is not required for components of this secretory program to be engaged; rather, cell stress and DNA damage are the central initiators. This concept broadens the description of these largely overlapping programs to include the acute stress-associated phenotype (37) and DNA damage-associated secretory program (DDSP). Deep discovery-driven analyses of transcript and protein responses to genotoxic stress induced by cancer therapeutics have identified several hundred factors deriving from benign cells comprising the tumor microenvironment (32, 38, 39).
The composition of the DDSP is complex and includes proinflammatory cytokines such as IL-6 and IL-8, extracellular matrix–altering proteases, proneurogenic factors, angiogenic growth factors, and epithelial mitogens that include agonists for the epidermal growth factor receptor (EGFR), such as amphiregulin and epiregulin (38, 39). These cell-nonautonomous effectors of the stress-response program likely evolved to propagate a tissue-damage signal locally and distantly in order to enhance the elimination of damaged cells through immune clearance, and hasten repair through angiogenesis and the migration and proliferation of epithelial and stromal cells. However, in the setting of a malignancy, where neoplastic cells co-opt such microenvironment cues, such effects may have adverse consequences. Individual components of the DDSP can suppress apoptosis and enhance the proliferation of premalignant and malignant epithelium (34), stimulate migration and invasion (38, 39), and transition epithelial cells to acquire mesenchymal phenotypes (38) with augmented resistance to chemotherapy and radiation (Fig. 1; ref. 40).

Findings from several preclinical studies support the concept that treatment-induced microenvironment damage can promote adverse tumor outcomes. Recent work using modern tools of molecular biology recapitulated the insightful studies carried out in the 1950s by Revesz (41), who enhanced the growth of transplanted allogeneic and syngeneic tumors by combining lethally irradiated tumor cells with nonirradiated tumor cells. This so-called Revesz effect was later shown to be due to the metabolic activities of the irradiated cells resulting in the production of diffusible factors that conditioned the tumor microenvironment (42, 43). More recently, using a mouse model of breast carcinoma, Nguyen and colleagues (44) determined that ionizing radiation acting on the breast microenvironment accelerated the development of aggressive p53-null breast cancers. The development of these tumors was found to be influenced by TGF-β signaling and exhibited distinct

Figure 1. Therapy resistance is promoted by genotoxic treatment-induced damage responses in the tumor microenvironment. The local environment in which most neoplasms originate is a complex ecosystem comprised of cancer cells, benign resident cells, and transient cells (e.g., inflammatory cell types), as well as additional structural and soluble components. Genotoxic cancer therapeutics induce DNA damage in tumor cells, leading to cell death or senescence, but can also induce genotoxic stress in benign cells, such as tumor-associated fibroblasts comprising the tissue stroma. DNA damage and other stressors initiate damage response programs, such as the DDR, with several effector arms, including the generation and secretion of a diverse spectrum of cytokines, growth factors, and proteases (here denoted the DDSP). Individual components of the DDSP are well known to promote inflammation, angiogenesis, epithelial-to-mesenchymal transition, and tumor cell proliferation, and to augment resistance to cancer therapeutics. Targeting individual DDSP components or key upstream master regulators (e.g., NF-κB, p38MAPK, and PARP) may enhance the effectiveness of commonly used antineoplastic agents by suppressing microenvironment-derived resistance mechanisms. BV, blood vessel; CC, cancer cell; EC, epithelial cell; IC, inflammatory cell; rCC, resistant cancer cell; SC, stromal cell.
molecular programs involving estrogen receptor and stem cell activity. Similar results were reported in studies of myogenic cells, in which implanted cells rapidly progressed to poorly differentiated tumors in irradiated muscle microenvironments relative to cells implanted into nonirradiated muscle (45). Tumorigenicity was also found to be dependent on the dose of preirradiation and to vary depending on the host's genetic background. Whether such damaged microenvironments would also promote therapy resistance has not been tested.

Studies using genotoxic chemotherapeutics have extended these observations to show that treatment-induced damage to the microenvironment can promote a chemoresistant niche of residual disease that subsequently serves as the nidus for relapse. In experiments using doxorubicin to treat the 4E-Myc model of transplantable lymphoma, Gilbert and Hemann (37) determined that the surviving metastatic tumor cells were exclusively localized to the thymus. Detailed molecular analyses of damage responses in different lymphoid tissues and of individual cell types comprising these tissues identified IL-6 and Timp-1 as prosurvival factors secreted selectively by thymic endothelial cells. Tumor-cell resistance was shown to be due to the paracrine production of IL-6 and Timp-1, and inhibition of these factors, or the upstream signaling pathway operating through p38 mitogen-activated protein kinase (p38MAPK), enhanced the effectiveness of subsequent chemotherapy (37). In addition to providing proof-of-principle that damage induced by cancer therapeutics to residents of the tumor microenvironment can influence tumor behavior (in this case, therapy responses), this study showed that different tissues, and indeed the distinct cell types that comprise these tissues, have varied damage responses, a finding that has important implications for designing clinical trials to exploit these results.

Clinical–Translational Advances

Therapeutic context

The concept of developing treatment strategies to modify the tumor microenvironment or interrupt interactions between tumor cells and components of the microenvironment is attractive for several reasons. First, this approach has been applied successfully in several malignant diseases, as exemplified most strikingly in multiple myeloma, where cotargeting the tumor microenvironment is now a mainstay of the overall treatment paradigm (20). Second, there are many potential ways to influence the tumor microenvironment to ensure more effective tumor-cell killing, ranging from mobilizing tumor cells [e.g., via CXCR4/CXCL12 axis blockade (46)] to breaking down desmoplastic barriers for more efficient drug penetration (15). Third, because the tumor microenvironment targets are generally derived from benign cells involving well-conserved developmental pathways, they are unlikely to be subject to mutation and resistance. Fourth, most tumor microenvironment targets represent a non–cross-reactive feature of the tumor that may not contribute substantially to toxicity.

In treatment-induced therapy resistance, it is important to consider that context is critical: The treatment itself can unmask or induce new opportunities for intervention. Unfortunately, the standard regimens that are currently used to treat most solid tumors are ideally suited to promote microenvironment-mediated resistance. Most chemotherapeutics are dosed in a sequence of treatment cycles that are generally designed to allow normal host tissues and organs to recover and avoid major morbidity and host lethality. Radiotherapy is similarly administered in a series of fractionated doses at intervals spanning days to weeks. Initial cycles of treatment can eliminate a substantial percentage of the tumor cell mass, but they can also induce a damage response in cells that constitute the tumor microenvironment (Fig. 1). Tumor cells that survive the first salvos of therapy are thus exposed to the high levels of growth factors, cytokines, and proteases that comprise the DDSP and are capable of bolstering the remaining tumor cells to survive subsequent treatment cycles. In the murine lymphoma studies described above (37), key prosurvival factors such as IL-6 and Timp-1 emerged as therapeutic targets only in the context of treatment, whereas without genotoxic stress, suppression of these factors was not relevant. The pro-growth, prosurvival, and proangiogenic components of the DDSP may also underlie the accelerated tumor repopulation kinetics that have been observed during intervals between treatments and account for rapid tumor repopulation, an important cause of treatment failure (47).

Cotargeting specific microenvironment effectors

The robust induction of growth factors and cytokines by DNA-damaging therapies may be a contributing factor in the limited responses observed in clinical studies of targeted therapeutics, such as those designed to inhibit angiogenesis or suppress EGF signaling. Following genotoxic treatments, small-molecule inhibitors and receptor-directed antibodies must contend with very high local treatment-induced concentrations of ligands for these receptors, such as VEGF, amphiregulin, and epiregulin. Recognizing that multiple distinct ligands may activate redundant signaling programs to resist these targeted treatments is the initial step in designing the appropriate clinical studies to confirm effective pathway suppression. Although it is remarkably diverse (36, 38), the secretory program induced by DNA damage and the attendant cell stress is not unlimited, and it is likely that only a subset of the program effectively contributes to therapy resistance. Further, components of the DDSP may assist in controlling tumor growth through host damage response signaling that attracts inflammatory cells and engages other tissue repair processes. Thus, a reasonable strategy would be to identify and cotarget only the key DDSP factors that are responsible for inducing a therapy-resistant phenotype.

Cotargeting the collective DDSP

An alternative to targeting individual resistance-promoting components of the DDSP, which may require multiple drugs deployed in combination to effectively suppress...
particular paracrine interactions, would be to inhibit the master regulators that transduce the DDR signal to modulate the expression of large subsets of effector proteins. Although this system is complex and incompletely understood, current knowledge about the DDR suggests several nodes that could be evaluated (Fig. 1). For example, inhibition of p38MAPK has been shown to suppress the secretion of most stress-responsive secretory proteins in fibroblasts (35), primarily through NF-xB, which is also an attractive target in the context of regulating DDR-induced responses. PARP-1 has been shown to be activated in response to DNA damage and to propagate a signaling cascade that includes the production of a secretome with protumoral and prometastatic properties (48). Thus, PARP-1 inhibitors, which are currently in clinical trials for the treatment of breast and ovarian cancers, could be repurposed for inhibiting the microenvironment DDSP in the context of genotoxic tumor therapy. IL-1α, itself a component of the damage-associated program, has been shown to promote the secretion of several key proinflammatory cytokines via interaction with cell-surface IL-1R and consequent activation of NF-xB (49). It is likely that additional master regulators of the DDSP will be identified as our knowledge about the signal transduction program matures.

Conclusions

Many questions remain regarding the optimal strategies for effectively suppressing the prosurvival microenvironment induced directly by cancer therapeutics. The extent to which damage-associated secretory responses vary among different organs, different cell types within tissues, and different individuals—and how this variation is controlled—remains unclear. Such information may be quite important when considering microenvironment targets in different primary tumor locations and sites of metastatic disease. The systemic effects of DNA-damage responses also likely influence the resistance of tumor cells to treatment. The duration or persistence of damage-associated paracrine activity has also not been established, and this may be quite important for designing clinical trials that sequence genotoxic agents with agents that inhibit microenvironment factors, and for understanding therapy-resistant niches and tumor-cell dormancy and reactivation. Intuitively, it seems that suppressing the damage response prior to genotoxic treatment would be ideal. Sequencing microenvironment agents between intervals of genotoxic therapy is also a reasonable approach and is analogous to metronomic designs in which cytotoxic agents are alternated with cytostatic drugs to inhibit rapid tumor repopulation (50).

It is becoming clear that context (in this case, the microenvironment) profoundly influences tumor-cell behaviors, including treatment resistance. Of importance, this biocircuitry is dynamic, and for every action, such as exposure to genotoxic stress, there are reactions and consequences throughout the micro- and macrosystems. Defining the interactions among tumor cells, benign constituents of the tumor, and the influences of treatment will likely yield more effective combinatorial strategies that improve upon conventional approaches that heretofore have focused primarily on the neoplastic cell.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: P.S. Nelson
Development of methodology: Y. Sun, P.S. Nelson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sun
Writing, review, and/or revision of the manuscript: Y. Sun, P.S. Nelson

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References

Damage-Induced Cancer Therapy Resistance


12. Williams RT, den Besten W, Sherr CJ. Cytokine-dependent imatinib resistance in mouse BCR-ABL+/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/-, -/