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Synergizing immunotherapy with molecular-targeted anticancer treatment

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The therapeutic opportunity for anticancer kinase inhibitors (KIs) that block cell-signaling pathways is materializing. Yet, these molecular-targeted therapies are not tailored to be allies of the immune system, and often antagonize it despite generating antigenic activity. KIs usually offer an incomplete cure and one culprit is the lack of synergy between the drug and the immune system, a problem that is magnified when the therapeutic context involves HIV-1-induced immunosuppression (AIDS). We outline a strategy to fulfill the therapeutic imperative of recruiting cooperative immune responses. Accordingly, we propose a method to redesign anticancer drugs to harness the antigenic products of drug-induced apoptosis of tumor cells, thus eliciting an adjuvant immune response.

Introduction

Small molecules such as kinase inhibitors (KIs) that interfere selectively with cell-signaling pathways represent a therapeutic opportunity in cancer treatment [1–11]. Promising as they are, most drug-based anticancer therapies are incomplete and do not provide a decisive cure [12]. A well-known culprit for failure in the long run arises from the somatic evolution of patterns of drug resistance that often materialize as site mutations. Such somatic mutations compromise the affinity of the drug for its target or increase the affinity for the kinase natural ligand ATP, in the case of ATP-competitive inhibitors [12]. A far less acknowledged culprit for the failure of drug treatment arises because these targeted therapies are typically not tailored to operate as an ally of the immune system, and often antagonize it despite generating antigenic activity [13–16]. Largely discovered through trial and error, KIs are often of limited applicability because drug treatments are marred by episodes of relapse and by the development of drug resistance and intolerance [12,17]. As said, one culprit for this incomplete success is the lack of synergy between the drug and the immune system, with the latter often incapacitated at crucial junctures owing to antagonistic effects generated by the drug [13,14,18–21]. Thus, a therapeutic requirement arises from the need to recruit cooperative immune responses concomitant with the molecular-targeted treatment. The goal is to design anticancer drugs that inhibit targeted cellular functions and steer the immune system to harness the antigenic products of the drug-induced apoptosis of tumor cells. To fulfill this need for therapeutic integration, we propose redesigns of anticancer drugs that fulfill three constraints: (i) nanomolar activity against anticancer targets; (ii) reversal of tumor-induced immunomodulation; and (iii) removal of drug-induced immunosuppressive activity.

The drug design strategies introduced to address the therapeutic imperative of immunosynergy have the potential to revolutionize cancer treatment and the understanding of the adaptive immune response by steering it with molecular-targeted therapy. We are counting on the premise that, by restoring the adaptive immune response to drug-induced antigenic activity, we shall be able to create synergies that will reciprocally empower the immune system and drug-based anticancer treatment. Novel possibilities to harness and manipulate the immune system will probably transpire from the evaluation of immunosynergic drugs.

Therapeutic shortcomings of anticancer drugs that suppress the adaptive immune response

Undesired cross-reactivity modulating the immune response

In practice, the level of molecular fine-tuning required to redesign an anticancer drug into an immunosynergic drug cannot be achieved within the drug discovery paradigm based on trial and

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error and high-throughput screening [17,22]. Rather, a rational design approach is needed [23]; an arena where novel molecular filters can be exploited to control drug specificity better [24,25]. Thus, an unprecedented control of specificity [17] is required to design therapeutic agents capable of discriminating between cancer-related targets and immunosuppressive targets. The reward in building immunosynergic molecular therapies through rational design is potentially immense, because these targeted therapies will have a formidable ally – the immune response – in their anticancer activity.

Well-established anticancer KIs like imatinib [1–3] or dasatinib [4–9] are also known to be directly immunosuppressive [13,14,18–21] through their powerful blockade of upstream signaling in the adaptive immune response and, yet, as shown in this study, they hold promise as chemical scaffolds that can be turned into immunosynergic drugs. The choices are justified because these KIs are nanomolar inhibitors of the major anticancer target c-KIT [2,3,8,9], the kinase of the stem cell factor (SCF) receptor, and hence the KI treatment is expected to have the additional effect of reversing tumor-induced immunosuppression, at least in certain tumor environments where tumor-secreted SCF is deployed to hijack the immune system [26]. The latter effect is promoted by the accumulation of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), the development and activation of which requires the SCF expressed by the tumor cells. However, imatinib and dasatinib are also direct immunosuppressants [13–15,18–21], and this role is clearly antagonistic to their anticancer activity, thus requiring careful removal through a molecular remodeling of the parent compounds using stringent selectivity filters [23–25].

More precisely, these KIs are powerful (nanomolar) inhibitors of two crucial kinases implicated as upstream signal transducers controlling the immune response: lymphocyte-specific protein tyrosine kinase (LCK) and colony-stimulating factor 1 receptor (CSF1-R) [17,22]. In fact, dasatinib is the most powerful inhibitor of LCK known to date (K_d = 0.2 nM) [22]. Specifically, LCK is a signal transducer for the signaling cascade that originates in the CD4 and CD8 receptors expressed on the surface of T cells in antigen-triggered T cell differentiation (http://www.genome.jp/kegg-bin/show_pathway?map=hsa04660&show_description=show) and in natural killer (NK) cell-mediated cytotoxicity (KEGG, release 8/2/2013, http://www.genome.jp/kegg-bin/show_pathway?map=hsa04650&show_description=show) [27]. By contrast, the CSF1-R kinase is implicated in the development of the monocyte/macrophage (M/M) lineage (http://www.genome.jp/kegg-bin/show_pathway?map=hsa04640&show_description=show) [27]. Hence, both KIs suppress antigen-specific T cell effector functions and NK cytotoxicity and inhibit the hematopoietic development of crucial components of the immune response.

Thus, imatinib and dasatinib are likely to hamper the adaptive immune response triggered by the strong antigenic activity they generate and compromise the antigen presentation by precluding macrophage development. The profound inhibition of all antigen-specific T cell effector functions at therapeutically relevant concentrations has been mechanistically tracked down to the early blockade of signal transduction events promoted by LCK inhibition [13,14]. In fact, LCK is central in the transduction of T cell receptor (TCR) signaling in response to MHC-I and MHC-II antigen presentation that ultimately promotes T cell differentiation and proliferation. Thus, the drug-induced immunosuppressive effects caused by LCK inhibition – purposely engineered in the case of dasatinib [28] – actually betray the purpose of these drugs as anticancer agents, possibly enabling the development of episodes of relapse and drug resistance and impacting the frequency of opportunistic infections. In the case of dasatinib, the original drug discovery pursuits seemed to focus squarely on modulating the immune response [28]. The repositioning of dasatinib as an anticancer agent seemed to have arisen as an afterthought, because the precursor drug imatinib showed cross-reactivity against LCK as well as the cancer-associated targets Bcr-Abl, c-KIT and platelet-derived growth factor receptor (PDGFR). Furthermore, the LCK-related immunosuppression by both KIs is reinforced by the blockade of macrophage development – and thereby antigen presentation – caused by CSF1-R inhibition (both compounds are nanomolar inhibitors of CSF1-R).

These immunoantagonistic effects are ostensibly at odds with the need to maintain an uncompromised immune response to fight cancer, unless the kinases targeted for immunosuppression also happen to be relevant anticancer targets. Thus, the immunosuppressive effects are likely to impede a lot of the ongoing efforts to apply these KIs to combat cancers other than hematologic malignancies, where immunosuppression could become adjuvant. Even in those applications for which FDA approval has been obtained [i.e. the treatment of chronic myelogenous leukemia (CML) and Philadelphia-chromosome-positive acute lymphoblastic leukemia (Ph+ALL); http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021588s024lbl.pdfatypical; http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm231409.htm], opportunistic infections have been reported in patients treated at therapeutic doses [19].

Potentially immunosuppressive anticancer drugs identified from kinome-wide screening

A kinome-wide screening of KIs [17,22] reveals that potential side-effects and immunosuppressive complications due to nanomolar inhibition of LCK (dissociation constant K_d <50 nM) are likely to be a concern for several compounds of therapeutic interest. Thus, a significant drug-induced impairment of adaptive immune responses, mostly affecting T cell and NK cell activation, is readily expected for the following KIs: NVP-AST487 (Novartis, K_d = 11 nM); nintedanib (BIBF-1120, Vargatef®, Boehringer-Ingelheim, K_d = 6.2 nM); crizotinib (Xalkori®, Pfizer, K_d = 30 nM); dasatinib (Sprycel®, Bristol-Myers Squibb, K_d = 0.2 nM); foretinib (GSK1363089, GlaxoSmithKline, K_d = 6 nM); imatinib (Gleevec®, Novartis, K_d = 40 nM); nilotinib (Tasigna®, Novartis, K_d = 47 nM); PD-173955 (Parke-Davis, K_d = 1.1 nM); bosutinib (SKI-606, Bosulif®, Wyeth/Pfizer, K_d = 0.59 nM); NVP-TAE684 (Novartis, K_d = 49 nM); and vandetanib (Caprelsa®, AstraZeneca, K_d = 17 nM) [22].

Although the anticancer activity is a desired clinical outcome for these KIs, the reported structural similarities between LCK and validated anticancer targets like c-KIT, PDGFR and Src-family kinases introduce extremely undesirable cross-reactivities. In fact, although these KIs are being actively evaluated as anticancer agents, their nanomolar inhibition of LCK truly compromises the immune system, depriving the patient of a key endogenous resource to fight the disease. This adverse aspect of treatment seems to have been overlooked (except in the cases of imatinib and dasatinib as indicated previously). Therefore, this review

advocates the need to address the importance of building a therapeutic alliance between immune response and molecular-targeted therapy. This goal could be achieved by exploiting a selectivity filter purposely created to guide the redesign of anticancer drugs to remove targets related to immunosuppression.

Toward redesigning anticancer drugs to make them immunosynergic

To redesign an anticancer drug to make it immunosynergic, an unprecedented level of control of drug specificity, well beyond the current drug discovery standard, is required. This is because anticancer activity must be retained while targets related to immunosuppression are selectively removed from the drug affinity profile. In this regard, we can exploit an effective selectivity filter to guide molecular redesign: known as wrapping [12,23–25,29]. Wrapping is a descriptor of the structural defects in the protein target, known as dehydrons, for a comprehensive review see [29]. Dehydrons are poorly packed – under-wrapped – intramolecular H-bonds and represent structure-disruptive local exposures of the backbone to the aqueous solvent. Two key properties of dehydrons make them selectivity-promoting features for the drug designer: (i) they are sticky sites because they promote the removal of surrounding water to enhance the H-bond stability; (ii) they are generally not conserved across proteins of common ancestry (homologs) [12]. The dehydron pattern of proteins is not conserved even in cases of very high structural similarity, as is the case between anticancer targets like c-KIT, PDGFR or c-Src, as well as immunosuppression-related targets like LCK.

Thus, the problem of target discrimination tailored to our therapeutic challenge could well exploit this new selectivity filter that can transcend structural similarity and take advantage of differences in the dehydron patterns of the target kinases (Figs 1,2). Thus, if a dehydron is present in an anticancer target protein (i.e. c-KIT) and the H-bond at the same location in a homologous off-target protein (i.e. LCK) is well-wrapped, we might redesign the original drug with its undesired cross-reactivity into a new drug that can differentiate the two targets, binding only to the desired target. The 'dehydron-wrapping' drug, whereby the drug improves the dehydron packing upon association, becomes an expansion of the original chemical scaffold and is intended to exclude water locally from the specific dehydron present in the desired target and absent in the undesired target at the same spatial location [12]. Specifically, the dehydron-wrapping capability of a drug is built by expanding the original parent compound with the addition of a nonpolar group (usually methyl) that can penetrate the desolvation domain of the preformed targetable dehydron upon drug–target association. Because the incorporation of a methyl group usually compromises bioavailability, a compensatory change increasing drug solubility is often introduced at an innocuous site removed from the protein–ligand interface [12].

To illustrate this operational strategy, let us consider the problem of redesigning imatinib into a new drug that retains anticancer efficacy against gastrointestinal stromal tumor (GIST) [2] and other c-KIT-dependent cancers (c-KIT dependent melanoma) [24] while becoming immunosynergic by selective removal of the anti-LCK activity of imatinib. The dehydron patterns for the imatinib-binding region of the desired target c-KIT (PDB accession code: 1T46) and the undesired target LCK (PDB accession code: 2PL0) are displayed in Figs 1,2, respectively. Of crucial importance, as guidance to imatinib remodeling, is the dehydron (green stick) pairing residues Cys673 and Gly676 in the nucleotide-binding loop of c-KIT (Fig. 1b). This dehydron becomes a well-wrapped H-bond (gray segment) pairing residues Met319 and Gly322 in the imatinib target LCK (Fig. 2). Thus, the uniqueness of the c-KIT Cys673–Gly676 dehydron guides us to redesign imatinib into a more selective dehydron-wrapping drug by expanding the chemical scaffold at the position highlighted in the terminal ring (Figs 1b,2). Thus, the resulting compound WBZ_4 [30] arises from methylation of imatinib at the position indicated in Figs 1b,2 and its chemical structure is given in Fig. 3. WBZ_4 is a dehydron-wrapping drug that stabilizes the dehydron 673–676 of c-KIT upon association with the target, whereas it unfavorably promotes water removal from the surroundings of the well-wrapped 319–322 H-bond in off-target

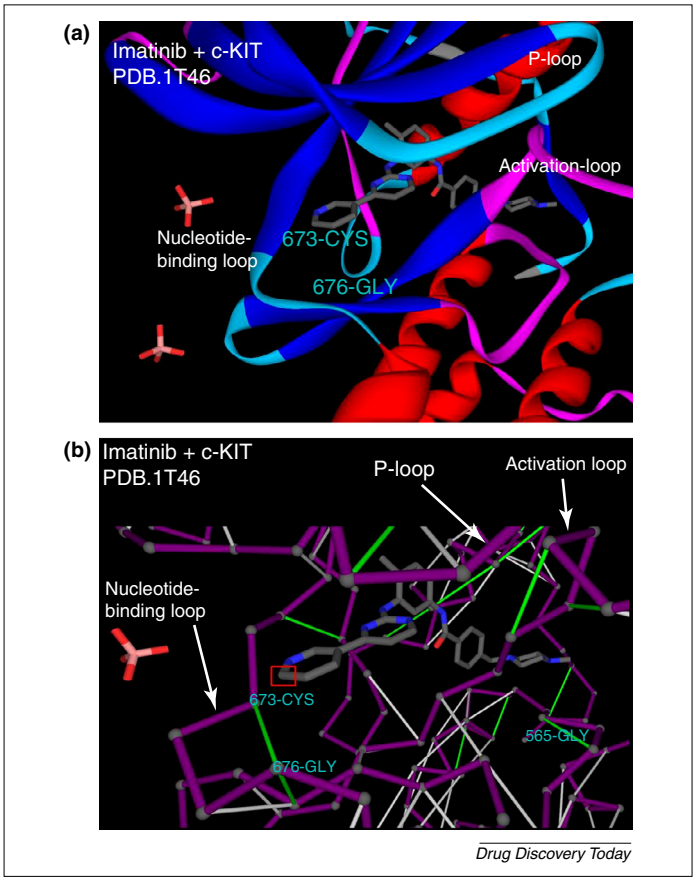

FIGURE 1

Structure and dehydron pattern of anticancer target kinase c-KIT in complex with kinase inhibitor (KI) imatinib (PDB entry: 1T46). **(a)** The protein is shown in ribbon representation colored conventionally according to the secondary structure motif (red = α-helix, blue = β-strand, light blue = turn, pink = loop); the drug is represented by bond-sticks colored conventionally according to chemical element (gray = carbon or hydrogen, blue = nitrogen, red = oxygen, pink = phosphorus). **(b)** Dehydron pattern for the protein in complex with the drug. The protein backbone is displayed in magenta, with virtual bonds joining adjacent α-carbons, well-wrapped (buried) H-bonds are represented by gray sticks joining the α-carbons of the paired residues and dehydrons are shown as green sticks. Of interest is the dehydron Cys673–Gly676 present in c-KIT that becomes a well-wrapped H-bond pairing residues Met319 and Gly322 in lymphocyte-specific protein tyrosine kinase (LCK). This selectivity feature unique to c-KIT directs the remodeling of the imatinib chemical scaffold by methylation at the location highlighted by the red rectangle.

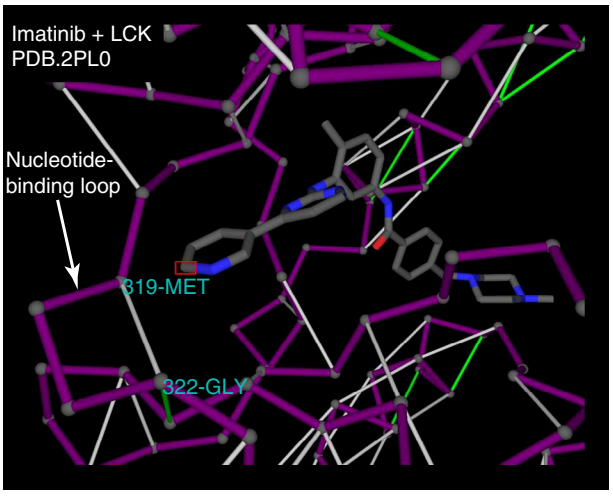

FIGURE 2

Dehydron pattern for lymphocyte-specific protein tyrosine kinase (LCK) in complex with imatinib with structural coordinates obtained from PDB entry: 2PL0. The representation convention use in Fig. 1b is followed. Upon structural alignment, the dehydron Cys673–Gly676 in c-KIT becomes the well-wrapped H-bond Met319–Gly322 in LCK.

LCK. Therefore, WBZ_4 retains nanomolar activity against c-KIT but shows no detectable inhibitory activity against LCK [25]. The immunosynergic properties of WBZ_4 await validation but its higher selectivity relative to the parent compound is indicative that there will be no impairment of the adaptive immune response triggered by the antigenic activity generated during drug treatment.

Similarly, a redesign of imatinib discriminating between desired target SCF receptor (via c-KIT) and CSF1-R is essential to secure development of the macrophage lineage during molecular therapy and could also be carried out by exploiting the dehydron filter. We can take advantage of local differences in the dehydron patterns and align this wrapping representation for PDB entries: 1T46 and 3LCO (for c-KIT and CSF-1R, respectively).

Preliminary evaluation of immunosynergic drug prototypes

The immunosynergic drugs should undergo testing at all levels, from *in silico* to clinical. Using the dehydron selectivity filter [12,30], the parent drugs can be reworked into compound series with nanomolar affinity toward the original cancer targets with immunosynergic properties arising from: (i) removal of activity against LCK (class A); (ii) removal of activity against the CSF1-R kinase (class B); (iii) simultaneous removal of both cross-reactivities (class C).

To test class A drugs, we can assay for TCR-mediated T cell proliferation and activation in a dose-dependent manner using the parent compounds in the control assays. Besides testing the redesigns in proliferation assays using cancer cell lines, we can test for selective removal of the inhibition of antigen-specific T cell effector functions. Purified CD4+ cells and virus-specific CD8+ T cells (naive and memory T cells) should be studied *ex vivo*, after antigen-specific effects are confirmed in defined T cell clones [31]. Functional outcomes including cytokine production, degranulation, activation, proliferation, apoptosis and/or necrosis induction and signal transduction mediated by LCK should all be assayed *ex vivo* on CD4+ and CD8+ T cells [31] treated with class A and class C variants of parent drugs and the results contrasted against those using parent drugs as controls. Immunoblot analysis of antigen-specific response should confirm that the massive inhibition by anticancer drugs of T cell activation, proliferation, cytokine production and degranulation is the result of blockade of early TCR-induced signaling transduced by LCK. Because the anti-LCK activity is specifically removed in class A and class C compounds, we expect to have restored all antigen-induced T cell functions. Thus, the class A compounds would be readily and ideally suited to be combined with immunotherapies that should harness the initial antigenic activity of the molecular-targeted therapy.

Class B compounds can be tested by examining the development of the M/M lineage from bone marrow progenitors at concentrations of class B or anticancer drugs of the order of 0.5–1 μM [26]. Based on the KEGG annotation cited above, we conjecture that anticancer drug impairment of M/M development is the result of drug inhibition of CSF1-R, so the impairment should vanish as the hematopoietic stem cells (HSCs) are treated with class B drugs. Class C compounds should be tested following all the protocols mentioned above and are expected to restore antigen-induced T cell response, M/M development and macrophage-mediated antigen presentation, hence they should be optimally suited to be combined with immunotherapy, because they are likely to restore cross-talk between

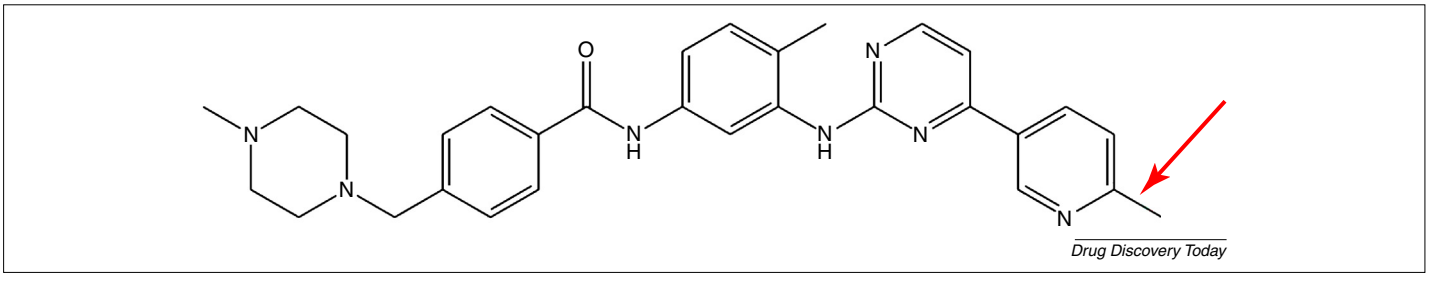

FIGURE 3

Chemical structure of compound WBZ_4, a nanomolar inhibitor of c-KIT that does not exhibit detectable anti-lymphocyte-specific protein tyrosine kinase (LCK) activity [25]. The red arrow indicates the methyl group incorporated as the only modification to the parent imatinib scaffold. To address bioavailability issues in therapeutic use, this compound will require further chemical modification at a site removed from the target/ligand interface. Such remodeling is currently being pursued by the author.

different immune responses. In all cases, we can corroborate retention or enhancement of anticancer activity as the parent drugs are molecularly re-engineered exploiting the dehydron filter, following the same *in vitro, ex vivo* and *in vivo* assays that have led to assert the therapeutic value of the parent drugs.

The novel targeted therapies will bring about significant antigenic activity by promoting apoptosis of tumor cells without compromising the immune response. This will make them amenable to be synergistically combined with immunotherapies. The choice of the latter will depend on the changes in the components of the innate and adaptive immune system caused by the targeted therapy and relative to the parent drug treatment. The immune response triggered by targeted therapy should be determined in animal models and the immunotherapies will probably include depletion of Tregs by treatment with CD25 antibody, targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) with monoclonal antibodies like ipilimumab (suited for c-KIT-dependent melanomas to be treated by class A variants of imatinib) [32].

The aggravating therapeutic context of HIV-1-induced immunosuppression

Probably the need for immunosynergic anticancer drugs is nowhere more pressing than in the context of viral cancers within AIDS, an immunosuppressive setting induced by HIV-1 [33]. Drug-induced immunosuppression would be a highly undesirable effect especially in this therapeutic context where the immune system is already compromised by endogenous factors triggered by HIV-1 infection. One of the primary cancers concurrent with HIV-1-induced immunosuppression is Kaposi's sarcoma, caused by a facilitated herpes viral infection [34]. As recently demonstrated, new lines of treatment to prevent the reactivation of Kaposi's sarcoma herpes virus and invasiveness of infected endothelial cells involve targeting the inflammation-related kinase Mitogen-activated protein fourth upstream of kinase 4 (MAP4K4) [34]. Direct inspection of the screening data reported in Refs. [17,22] enables us to identify two very powerful nanomolar inhibitors of the MAP4K4 kinase: NVP-AST-487 (Novartis, $K_d = 15$ nM) and bosutinib (SKI-606, Bosulif®, Wyeth/Pfizer, $K_d = 8.2$ nM). Yet, none of them should be used in this specific therapeutic context because they are also extremely powerful inhibitors of LCK ($K_d = 11$ nM and 0.59 nM, respectively), as already noted in this review. Thus, to treat Kaposi's sarcoma in HIV-1-positive patients one must first attempt a wrapping-based molecular remodeling of both inhibitors to retain nanomolar activity against MAPK4K but suppress as much as possible their anti-LCK activity. This molecular re-engineering would entail assessing the differences in the dehydron patterns for the on-target kinase MAPK4K *versus* the off-target kinase LCK, and redesign the parent chemical scaffolds to discriminate the two proteins according to the premises of the wrapping technology [12].

Concluding remarks

Promising as they are, most drug-based anticancer therapies are incomplete and do not provide a decisive cure. Molecular-targeted therapy aimed at blocking cancer-related signaling pathways seldom recruits the immune system but rather antagonizes it. This problem is magnified when the therapeutic context for cancer treatment involves HIV-1-induced immunosuppression, as is the case with AIDS patients. Thus, we make it a therapeutic imperative to recruit cooperative immune responses triggered by molecular-targeted treatment. The main challenge posed in this review is to engineer drugs that inhibit targeted cellular functions without adverse interference, steering the immune system to harness the antigenic products that the drugs generate. To achieve this synergy we need to remove immunoantagonistic effects generated by the drug treatment while retaining the anticancer potency of the drug. This goal demands a paradigm shift in drug design.

Through their inhibition of anticancer target c-KIT, drugs like imatinib or dasatinib are likely to elicit antigenic activity because they induce apoptosis of tumor cells, and evidence suggests that c-KIT inhibition can reverse tumor-induced immunosuppression. Thus, these inhibitors partially fulfill the therapeutic imperative. Yet, because they are also nanomolar inhibitors of LCK and CSF1-R, they suppress antigen-specific T cell effector functions and the development of the macrophage lineage, hampering antigen presentation. Ultimately, these drugs compromise the immune response, partially defeating their own purpose. Besides imatinib and dasatinib, this review reports on nine additional compounds of clinical oncologic interest that are also likely to impair the adaptive immune response precisely at the juncture when it is most indispensable.

In consonance with the importance of therapeutic integration, this review advocates the design of immunosynergic anticancer drugs. The synergic designs must fulfill three constraints: (i) nanomolar inhibition of anticancer targets; (ii) reversal of tumor-induced immunosuppression; and (iii) removal of drug-induced immunosuppressive activity. To fulfill these goals, an unprecedented level of control of drug specificity is needed. The review advocates the exploitation of an effective selectivity filter to guide molecular design: wrapping. Wrapping is a descriptor of the unique structural vulnerabilities in a protein target and enables a level of control of drug specificity above and beyond structure-based design. The therapeutic challenge of immunosynergy requires this new generation of selectivity filters for target discrimination. The proposed strategy has the potential to revolutionize anticancer treatment and the understanding of the adaptive immune response by steering it along with molecular-targeted treatment. We are counting on the premise that restoration of the adaptive immune response triggered by drug-induced antigenic activity will ultimately empower drug-based anticancer treatment. Novel possibilities for therapeutic manipulation of the immune system are likely to arise from the evaluation of the emerging immunosynergic drugs.

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