"Molecular Targeting in Oncology"

Gleevec and the emergence of chemotherapeutic drug-resistant mutations

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Abstract

Gleevec is an important, new, molecularly targeted, anti-cancer agent that has demonstrated clinical efficacy in chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST). These malignancies develop after constitutive activation of Abelson (Abl) or Abl-like tyrosine kinases; Gleevec is a specific chemical inhibitor of such kinase activity. Many CML and GIST patients have relapsed while on Gleevec treatment, however. In this Chapter, I discuss why the emergence of resistance to Gleevec chemotherapeutic intervention is in retrospect neither surprising nor insoluble. Principles previously elucidated in the development of multidrug resistance in human immunodeficiency virus infections may prove useful in the approach to the next generation of targeted molecular therapeutics for CML. In general, multidrug protocols and agents targeted to multiple Abl sites simultaneously are likely to have a greater chance of success than single agent therapy.

Keywords: Gleevec, imatinib, STI-571, drug resistance, cancer chemotherapy, chronic myelogenous leukemia, gastrointestinal stromal tumor, BCR-ABL, structural biology

1. Introduction

Unquestionably, the most exciting success story of new targeted therapeutics has concerned the Abelson (Abl) kinase inhibitor Gleevec. This therapeutic agent, formerly called STI-571, and carrying the official name imatinib mesylate, was first developed for chronic myelogenous leukemia (CML) and later applied to gastrointestinal stromal tumor (GIST). Gleevec originated twenty five years ago at the pharmaceutical company Ciba Geigy, now Novartis, as a candidate inhibitor of protein kinases. Gradual refinements in structure restricted its specificity first to tyrosine kinases and then to a very limited set of tyrosine kinases that include primarily the Abl kinase family (v-Abl, c-Abl, $p185^{BCR\text{-}ABL}$ and $p210^{BCR\text{-}ABL}$, important in CML) and others with binding site architecture similar to Abl (Kit and platelet-derived growth factor receptor α and β forms, important in GIST). The excitement surrounding this agent arose in 1998, when Phase 1 clinical trials in CML patients showed dramatic improvements and very good tolerance of the drug. Phase 2 and 3 trials confirmed these results. Scientific reports have been numerous (2724 Medline citations as of April 2006) and many excellent and comprehensive reviews have been written (655 to date). However, despite the early success of Gleevec treatment, resistance to the drug began to be reported very quickly, which is not surprising, given the basic principles of mutation selection in single-agent therapy. It now seems to have been premature to think of Gleevec as curative, and the race is on for new derivatives that will overcome the problem of resistance. It seems wise at this stage to reflect on basic principles of chemoresistance and invest effort in rational strategies to continue the development of this agent and its analogs or derivatives.

2. Chronic Myelogenous Leukemia

CML is a relatively common adult hematologic neoplasm, and occurs rarely in children. Based on incidence rates from 2000-2002, approximately 1 in 619 men and women will be diagnosed with CML during their lifetime. Five-year relative survival rates by race and sex are 37.6% for white men, 41.2% for white women, 33.9% for black men and 35.3% for black women (1). The only well-characterized risk factor is exposure to ionizing radiation (2). CML has three phases. The malignancy typically presents with a long "chronic phase" that can last years, with mild symptoms. By definition, in this phase, 5% or fewer of the cells in the peripheral blood or bone marrow are blasts (immature cells of the myeloid lineage). The chronic phase is followed by an "accelerated phase", in which these compartments are populated with 6% to 30% blasts, and then a terminal "blast phase", wherein the fraction of blasts exceeds 30%. If additional clinical signs are present, such as splenomegaly or fever, the phase is termed "blast crisis". Untreated, the blast crisis is fatal.

Increasing severity of the disease and deteriorating prognosis is associated with the appearance of cells of the leukemic clone that characteristically contain a reciprocal chromosomal translocation involving the p arms of chromosomes 9 and 22, called the Philadelphia (Ph) chromosome. Measures of disease progression or responses to therapy therefore consider both molecular and cytogenetic characteristics, as well as clinical signs. Molecular assessments typically include reverse transcriptase (RT) treatment of peripheral blood cell RNA, followed by amplification of the transcribed BCR-ABL message by polymerase chain reaction (PCR). Typical detection limits are one cell in 10^5 (3). Cytogenetic assessments require viable bone marrow cells or more than 10% blasts in the peripheral blood to visualize

metaphases. Fluorescence *in situ* hybridization of the t(9:22) translocation junction has become an important diagnostic tool (4,5).

The t(9;22) reciprocal translocation creates a chromosomal fusion between the *BCR* gene, which stands for "break point cluster" and the *ABL* gene, termed Ph^+, leading to localization of the resultant protein to the cytoskeleton and unfettered tyrosine kinase activity in the Abl protein kinase domain. The fusion protein has many unregulated functionalities, most potently, an elevated and constitutive protein-tyrosine kinase activity, but also aberrant initiation of mitogenic signaling cascades that lead to uncontrolled growth in CML and GIST, and recruitment of downstream effectors of cell survival. Reduced apoptotic signaling is thought to be a uniquely important contributor to CML (6). The key role of the Bcr-Abl tyrosine kinase in CML etiology makes Bcr-Abl an appealing target for rational drug design. Nevertheless, there is a CML patient subpopulation that does not show evidence of Ph^+ abnormality, and for this situation, no molecular mechanism of leukemogenesis is known. It is possible that the early Ph^- stage of CML initiates a specific kind of genetic instability that may involve the Ataxia Telangiectasia (ATM) protein (7), leaving the *BCR* and *ABL* genes especially prone to translocation. Such genetic instability may promote the occurrence of Gleevec-resistant Abl mutations even before exposure to Gleevec in some CML cases (8). Epistatic factors probably also contribute to the individual-level variation in this instability stage. Once the translocation event has occurred, however, the abnormality is irreversible and progression is inevitable.

The advent of combination chemotherapy has extended lifespan for many hematologic malignancies. To appreciate the significance of this fact, one need only consider that in 1970, a person diagnosed with non-Hodgkin's lymphoma could expect to live only about a year, whereas today, combination chemotherapy and targeted molecular agents such as Rituximab, a

humanized monoclonal antibody chimera against the CD20 receptor on lymphoma cells, have boosted survival and been curative in many cases. Similarly, CML was considered incurable and fatal until the 1980s. Traditional therapies for CML have improved overall survival; high dose chemotherapy, donor lymphocyte infusion, stem cell transplant and biologic therapy, such as α-interferon, remain important therapeutic avenues.

The active site of the Abl protein kinase is conserved among related protein tyrosine kinases and has been well mapped and understood in structural studies (9,10). The kinase shifts between active and inactive conformations with the movement of a three-dimensionally unstructured "activation loop," dependent upon its phosphorylation state (11). Tyr^{393} is the site of phosphorylation within the activation loop of Abl (9) and Tyr^{823} is the site of phosphorylation within the activation loop of Kit (10). Phosphorylation appears to stabilize the active conformer of the activation loop. Gleevec, with its structure based on a 2-phenylaminopyrimidine core (Fig. 1), functions as a competitive inhibitor of ATP and is able to bind only to the inactive conformation, freezing movement of the loop and thus interrupting the catalytic cycle. The drug provides a high degree of specific inhibition, while being essentially inactive against serine-threonine protein kinases and most other tyrosine kinases. The 6-methyl substituent of the phenyl aniline moiety (Fig. 1) forms a hydrogen bond with Thr^{315} in the Abl active site (12), as does the nearby secondary amine (9). The important 6-methyl residue seems to be a primary determinant of the specificity of Gleevec for the Abl-related family of kinases, whereas a benzamide group at the phenyl ring is a determinant of activity against the platelet derived growth factor receptor (2). Several other kinases for which Gleevec has weak inhibition constants harbor a bulky nonpolar amino acid at this position, instead of threonine, which probably excludes the Gleevec molecule due to steric hindrance in the binding pocket. Hydrogen bonding between Gleevec and Thr^{315}

clearly identifies a central requirement for Gleevec's ability to inhibit Abl (9). Indeed, as will be discussed below, the substitution of isoleucine for this threonine accounts for a plurality of the reported Gleevec-resistant point mutations in the Abl active site.

Gleevec was Federally approved to treat CML in May 2001 based on the remarkable results of three clinical trials. Brian J. Druker, M.D., of Oregon Health Sciences University, deserves the lion's share of the credit for proof-of-concept and for shepherding Gleevec through the approval process. Gleevec is approved for treatment of patients that have progressed from the manageable chronic phase of the disease to the acute "blast crisis" phase, which is frequently fatal, and is useful in early stages of CML, as well as in GISTs (discussed below). Clinical trials of Gleevec in combination with other agents are ongoing. Complete hematological response (CHR) is defined as the normalization of the blood counts and the white cell differential, and the alleviation of all clinical signs. Complete cytogenetic response (CCR) is defined as no detection of Ph^+ metaphases. Major cytogenetic response (MCR) is defined as the detection of less than 35% Ph^+ metaphases. Molecular remission is defined as no BCR-ABL mRNA detectable by RT, coupled to PCR amplification (2). These negative definitions obviously require appropriate statistical power and controls, lest failure to detect derive from failure of the assay.

3. Gastrointestinal stromal tumors

The most frequently occurring gastrointestinal tumor in humans is gastrointestinal stromal tumor (GIST). These malignancies are thought to derive primarily from activating mutations in the *KIT* gene (13), which encodes a receptor tyrosine kinase. Originally, Kit was named Stem Cell Factor receptor (14). The kinase autophosphorylates on tyrosine, then signals through downstream effector pathways to promote proliferation (15), tumorigenesis, adhesion

and differentiation (16). Most GISTs harbor Kit mutations, but 3% harbor mutations in the platelet derived growth factor receptor α form (17), the active site structure of which is closely related to Kit and is also inhibited with Gleevec, as mentioned above. Treatment with Gleevec has clearly been of great value for GIST patients, given their poor prognosis: median survival after resection of the tumor is 15 months without Gleevec (18), but current two-year overall survival for patients treated with Gleevec is 70% (19).

4. Chemoresistance

In clinical trials, most patients treated with Gleevec responded, but relapse was seen in about 80% of individuals successfully brought into remission. In every relapsed patient studied, the level of Bcr-Abl kinase activity was elevated to pre-treatment values. Most interestingly, the common T315I point mutation in the active site eliminated Gleevec binding but did not compromise kinase activity (9). Mutations of Y253, E255, T315 and M351 in Bcr-Abl account for approximately 60% of those detected at the time of relapse (20). Goldman and Melo (21) have reported that in a sample of 179 Gleevec-resistant patients, 114 mutations were detected, and some patients had more than one mutation in the resistant CML clone; most of these were in the tyrosine kinase domain of Abl.

The mechanisms of genetic instability in CML clonal expansion (22) are not well understood, but are likely to involve rates of point mutation that are much higher than the background rate. Such instability therefore provides a central and essential factor for the rapid emergence of chemoresistance. Of particular seriousness, Fabarius *et al.* (23) used centrosome immunostaining and conventional cytogenetics to reveal that Gleevec treatment of normal fibroblasts (from human dermis, Chinese hamster embryo or Indian muntjak) causes dose-

dependent centrosome and chromosomal aberrations, independently of species. Thus, Gleevec treatment *per se* is likely to exacerbate the accumulation of genetic lesions.

5. Historical perspective

The phenomenon of biological resistance to chemical agents is well known and has been widely reported in fields as diverse as insect control with pesticides and antibiotic control of microorganisms, especially tuberculosis. Primary resistance refers to innate or natural ability to resist an agent, and is of marginal interest here. Secondary, or acquired resistance, arises from the biological processes of selection under the pressure of exposure to an agent and is a significant medical problem. Mutation of specific genes within a microorganism that are responsible for the transport or metabolism of the drug, or the signaling environment within the organism, enables the acquisition of drug resistance, often with dire consequences for the health and survival of a human host infected with that microorganism (24).

The elements required for the appearance of stable resistant clones of CML cells are: a mechanism for the introduction of frequent mutations, DNA replication to “stabilize” and perpetuate the mutations, the possibility that adventitious mutations exist and selective pressure to provide a proliferative advantage to the cells that harbor the adventitious mutations (25). It should be apparent that each of these elements is in place in the setting of CML under the conditions of Gleevec single agent therapy.

6. The case of methotrexate

The well known antimetabolite methotrexate has been in use for many years in treatment of acute leukemia and other neoplasms (26); it is often prescribed in combination with other

antimetabolites such as 6-mercaptopurine or 6-thioguanine. Methotrexate came to prominence in 1956 when it was used successfully to achieve the first cure of a metastatic malignancy, a choriocarcinoma. However, it soon became clear that resistance to this first line agent could pose a problem for successful therapy. The five main mechanisms of resistance to methotrexate treatment are: 1) amplification of the gene that encodes dihydrofolate reductase (*DHFR*), the protein product of which methotrexate is a competitive inhibitor; 2) increased cellular export of methotrexate by the multidrug resistance transporter or P-glycoprotein; 3) decreased cellular import of methotrexate; 4) mutation in the active site of dihydrofolate reductase in order better to discriminate between methotrexate and folic acid, the natural substrate of dihydrofolate reductase; and 5) decreased polyglutamination of methotrexate, which causes reduced cellular retention of methotrexate.

Each of these general classes of resistance mechanism but the last has now been identified in connection with resistance to Gleevec: 1) amplification the *BCR-ABL* gene has been reported (27) as a frequent mechanism of resistance; 2) the P-glycoprotein (28) and multidrug resistance transporter (29) have been implicated in Gleevec resistance and RNAi against the P-glycoprotein can confer Gleevec sensitivity to resistant CML cells (30). 3) Furthermore, variable expression of influx transporters such as hOCT1 has also been shown to be involved in resistance (31,32). GIST cell lines are likely to alter influx and efflux transporters under Gleevec selection (33). 4) As discussed above, point mutations that disrupt the binding of Gleevec to Bcr-Abl protein are numerous (34) and are among the most widely reported of resistance phenomena. Secondary mutations in Kit after treatment of GIST with Gleevec are also now reported; one study categorized up to four newly acquired *KIT* mutations in 14 patients (43.8%) (35). In

addition, 62.6% of GISTs that exhibit activating mutations in the platelet derived growth factor α form show point mutations associated with resistance to Gleevec (181 out of 289 cases) (36) .

In short, the well known mechanisms of chemotherapy resistance discovered over many years of methotrexate therapy now plague Gleevec therapy; molecular remission in CML is almost never reached through Gleevec treatment alone (34). The only consistently successful curative treatment of CML has been high-dose chemotherapy followed by allogeneic bone marrow or stem cell transplantation (37). Indeed, the exploration of Gleevec resistance is currently following a well established historical pattern (Fig. 2). Realism should have prevailed at its introduction, rather than enthusiastic assertions that Gleevec would be a revolutionary, spectacularly effective cure as a single agent (38,39). The notion that "all malignancies will depend on specific genetic defects and that it is simply a matter of defining the genetic lesion in each malignancy" (40), particularly for early stages when molecular defects are more uniform and potentially easier to correct or inhibit, is highly optimistic. Nevertheless, Gleevec should most realistically be thought of neither as outlier nor as startling new paradigm, but subject to all the biological rules of chemoresistance that have been painstakingly developed during the study of methotrexate.

7. The experience of anti-retroviral agent development for HIV.

Several principles have been developed to manage the troublesome occurrence of mutations in human immunodeficiency virus-1 (HIV) RT, which may be of value if applied to the case of chemoresistance in CML. An important principle in the treatment of HIV with chemical agents is the simultaneous introduction of combinations of anti-viral drugs that have not been used previously for a particular patient (41). In CML, partial cytogenetic or hematologic

responses are actually exceedingly dangerous states, because ongoing genetic instability in the surviving CML clones provides an ideal mechanism for the strong selection of resistant mutations under the pressure of a single anti-cancer drug. Therefore, the repopulation of the marrow and peripheral blood with resistant clones is almost inevitable. Deininger and Druker (2) have acknowledged that Gleevec's selective pressure favors the outgrowth of pre-existing resistant clones, similar to a bacterial culture treated with a single antibiotic. However, the pace of basic research into drug design for the next generation of chemical inhibitors of Abl suggests that there will soon be a respectable arsenal of agents available for physician choice, such as AP23464 (Ariad Pharmaceuticals), BMS-354825 (Bristol-Myers Squibb;42,43), SKI606 (Wyeth), PD180970 (Parke Davis), CGP76030 and AMN107 (Novartis; 43), VX-680 (Vertex Pharmaceuticals/Merck;44). Other targeted agents such as SU11248 (Pfizer), have value against Kit and platelet derived growth factor receptor α form (16).

The most productive places in the Bcr-Abl molecule to design new targeted therapeutics, to be used in combination with Gleevec, are probably outside the active site, and are likely to involve inhibition of movement of the activation loop. The novel investigational agent BMS-354825 (dasatinib) is effective against some resistant forms of Bcr-Abl (45,46), but not the T315I point mutant form (47). On the other hand, VX-680 has been successful in the treatment of Gleevec-resistant patients who harbor the T315I mutation, in which cases, unlike Gleevec, VX-680 binds to the active form of the kinase. VX-680 inhibits the kinase activity of both wild-type (K_i 68 nM) and T315I Abl (K_i 114 nM), but Gleevec inhibits the activity of only wild type Abl (47). Given the principles derived from experience with inhibitors of HIV RT, VX-680 and similar novel agents that bind independently of Gleevec, yet cooperate with Gleevec to stabilize

the Abl activation loop, might be good examples of a suitable first-line combination therapy and may work exceptionally well together to minimize chemoresistance.

Absent successful allogeneic stem cell transplantation, it is likely that clinicians will be forced to confront the problem of invariable relapse in CML, and will continue to depend upon chemical agents. The experience of HIV anti-viral drug resistance suggests the following analogous principles should be applied to CML therapy:

1. Several combinations of Abl-directed drugs should be used simultaneously, even if specificity is not optimal, provided that the combination can be tolerated.
2. Patients should be carefully monitored for the emergence of resistance, and new agents quickly substituted as they become available.
3. If a combination treatment protocol fails, it is important to change more than one component of the protocol. Substitution of a single agent, even Gleevec, may promote resistance to new agents by increasing the strength of the drug selective pressure.
4. Single agent therapy, such as Gleevec alone, should be avoided, given the obvious risk for the development of drug resistance.

Our molecular understanding of the Abl active site is still evolving, and the principles by which mutations are selected are not yet completely understood. However, it is conceivable that certain combinations of drugs will challenge the Abl protein with an insoluble problem: like HIV RT, it may be impossible for Abl to mutate to overcome combination drug inhibition, because of structural constraints within the protein. This feature made possible the success of "triple combination" therapy for HIV infection (48). As Mangel *et al.* (49) have proposed:

> "Combination therapy in the treatment of viral infections in which, for example, three different drugs against three different targets on three independent proteins are administered, has been highly successful clinically. However, it is only a

> matter of time before a virus will arise resistant to all three drugs, because the mutations leading to drug resistance are independent of each other. But, what if the mutations leading to drug resistance are not independent of each other, but confer some cost to the virus? If the cost is too great, than (sic) resistance may not arise. To impose such a cost in the clinical treatment of viral infections, we propose a new form of combination therapy. Here, three different drugs against three different targets on the same virus-coded protein are administered. If the physiological functions of the three different target sites are not independent of each other, then, a mutation at one site may alter the physiological functions at the other sites".

The observation in GIST cases that there was never more than one new mutation in the same sample from patients treated with Gleevec may bode well for such multiagent therapy that is targeted to a single protein (35).

8. Conclusions and lessons for rational drug design

Hirota and Isozaki (13) have pointed out that both CML and GIST appear to be special cases, because a single genetic activating event, such as translocation to produce *BCR-ABL* in the former case and mutation in *KIT* or *PDFGRA* in the latter, is necessary and sufficient to drive carcinogenesis. Relatively simple genetic lesions that account for neoplastic transformation are rare, however, and do not characterize the cancers to which the majority of morbidity and mortality in the United States may be attributed, such as breast cancer. Genetic lesions in the *BRCA1* and *BRCA2* loci increase the risk of breast cancer to about 80% for individuals with a family history, but account for only about a tenth of the cases that occur sporadically. Yet sporadic breast cancer is diagnosed in about 190,000 women in the United States annually, with a mortality rate approaching 20%. Multiple genetic lesions, including loss of tumor suppressor function; cytogenetic abnormalities and epigenetic factors almost certainly cooperate to create the tumorigenic environment within breast ductal tissue. Furthermore, another diverse set of

genes and epistatic factors is likely to control the invasiveness or metastatic potential of the primary tumor. It is unreasonable to expect that a single chemical agent will ever be sufficiently robust to inhibit such a complex and multifactorial process, no matter how successful single agents may be in special cases.

It is with the aforementioned molecular principles in mind that new research into the next generation of chemical inhibitors should be undertaken, and clinical trials designed for multiagent combination chemotherapy of CML and GIST.

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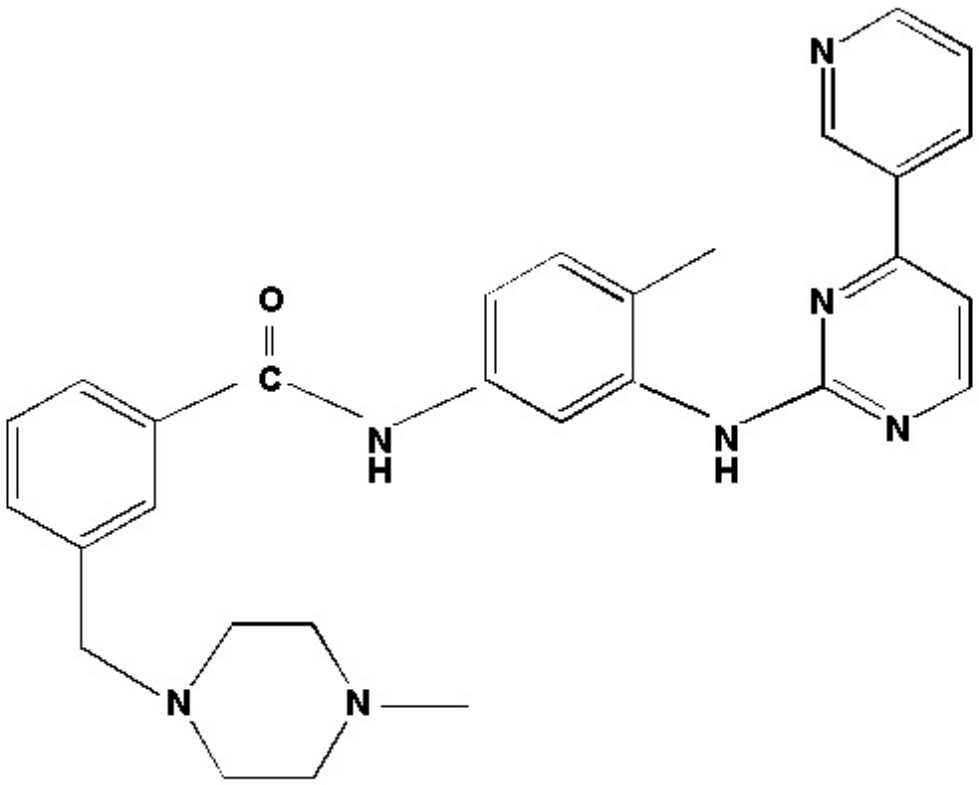
Figure Legends

Figure 1. *The chemical structure of Gleevec.* Note the predicted highly hydrophobic property of the molecule.

Figure 2. *Reported chemoresistance in two anti-cancer agents.* Medline citations reported by year, obtained through online database search of the years 1969 to 2005 (the last year for which complete citation information is available), for the two molecules methotrexate (filled circles) and Gleevec (open circles). Search terms were “methotrexate resistant” and the sum of “Gleevec resistant”, “imatinib resistant” and “STI-571 resistant”. There are no citations for Gleevec before 2001, because the compound was previously undiscovered.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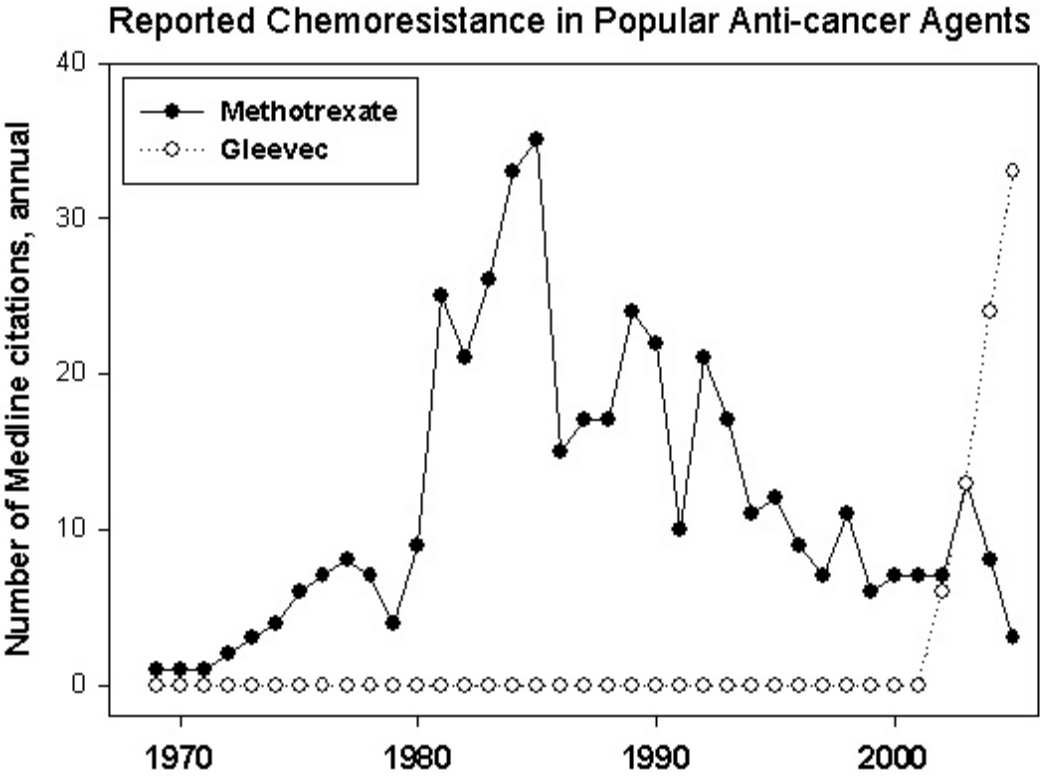
Reported Chemoresistance in Popular Anti-cancer Agents
Number of Medline citations, annual
Methotrexate
Gleevec
40
30
20
10
0
1970
1980
1990
2000