Preclinical Development

Real-time Fluorescent Resonance Energy Transfer Analysis to Monitor Drug Resistance in Chronic Myelogenous Leukemia

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Abstract

Despite the initial effectiveness of oncogene-directed cancer therapeutics, acquired drug resistance remains the ultimate “Achilles’ heel” for long-term durable remission in cancer patients. Acquisition of drug resistance is not more evident elsewhere than in the use of tyrosine kinase inhibitors, imatinib and dasatinib, for patients with chronic myelogenous leukemia. Hence, even though imatinib initially produces remission in the chronic phase, ultimately these therapeutics fail via the emergence of drug resistance, in which chronic myelogenous leukemia could inevitably progress to a terminal blast phase culminating in fatal outcome. Technically, it is challenging to predict the onset of drug resistance in a small number of oncogene-transformed cells, making the decision of when and how to employ second-generation tyrosine kinase inhibitors, or employ novel compounds that would be of benefit in treating drug-resistant Bcr-Abl mutants mainly retrospective. Here, we characterize a rapid and sensitive real-time fluorescent resonance energy transfer–based assay that is able to detect the in vivo activity of Bcr-Abl and its inhibition by small molecule compounds. Due to its real-time and in vivo nature, such an approach has the potential to monitor a drug-resistant phenotype, as well as to identify pharmaceutical agents that inhibit drug-resistant Bcr-Abl oncoproteins in vivo. Mol Cancer Ther; 9(11); OF1–9. ©2010 AACR.

Introduction

Chronic myelogenous leukemia (CML) is a multi-phasic myeloproliferative disorder composing ∼20% of adult leukemia and represents the prototype of a human cancer responsive to oncogene-targeted therapeutics (1). CML is usually diagnosed during a routine medical visit while in an initial chronic phase, which is cytogenetically characterized by clonal expansion of granulocytes and is well managed with currently available tyrosine kinase inhibitors (TKI; ref. 2). However, the mean duration of the chronic phase is 3 to 5 years and is followed by a less well-defined accelerated phase that ultimately leads to the terminal blast phase, in which there is an apparent reversion to earlier lymphoblast and myeloblast progenitors (2). Unlike the chronic phase, the blast phase is often refractory to treatment and can be fatal within 3 to 6 months (3). It is believed that the accumulation of secondary mutations gives rise to the switch from the chronic phase to the blast phase (4), although the epigenetic factors that drive blast crisis are not completely understood.

The driving force behind CML is the fusion protein Bcr-Abl produced by the Philadelphia chromosome (2). This results from a translocation between chromosome 22, which harbors the Breakpoint cluster (Bcr) gene, and chromosome 9 containing the c-Abl gene (5). The translocation produces a constitutively active version of the c-Abl tyrosine kinase in the form of Bcr-Abl (5). Inhibition of Bcr-Abl activity is currently the main form of therapy, which employs TKIs such as imatinib mesylate (Gleevec; refs. 6–8) and the more recent dasatinib (BMS-354825; refs. 9, 10). Although such approaches to the treatment of CML are efficacious in the earlier stages of the ailment, patients eventually become refractory to drug therapy and succumb to the disease, often due to mutations in the kinase domain, although some patients, harboring more detrimental mutations such as T315I, exhibit primary resistance to all currently available forms of therapy (11, 12).

Clinical problems associated with acquired resistance to imatinib have prompted the development of novel ATP-competitive small molecule TKIs with activity towards clinically relevant Bcr-Abl mutants (13–17). Given the remarkable progress in the development of second-generation TKIs for imatinib-resistant CML, such as dasatinib (9, 10) and nilotinib (18), there is an emerging need for in vivo technology that can rapidly and sensitively monitor Bcr-Abl tyrosine kinase activity and guide clinicians into decisions as to when and how to switch...
therapeutic modalities and evaluate resistance when it occurs in vivo. With this background, we set out to develop a fluorescent resonance energy transfer (FRET)–based assay using the Picchu biosensor (19) capable of detecting in vivo Bcr-Abl activity and inhibition by TKIs. We show here that Picchu is remarkably labile and responsive to Bcr-Abl activity in vivo, becoming inhibited within minutes of TKI administration, and hence, highly adaptable for real-time kinetics. Moreover, Picchu is highly sensitive to both the therapeutic inhibition of wild-type (WT) Bcr-Abl as well as to the drug resistance of clinically relevant Bcr-Abl mutants. Therefore, we posit that the Picchu FRET biosensor will have two major utilities—the first for pharmaceutical use in the screening of novel compounds that have high intracellular activity in suppressing clinically relevant Bcr-Abl mutations, and second, as an important clinical tool for diagnosing relapse determination of a drug-resistant phenotype because unlike currently available diagnostic methods like fluorescence in situ hybridization, which detects the Bcr-Abl fusion oncogene, Picchu FRET detects active intracellular Bcr-Abl, an important distinction because the Bcr-Abl gene has been detected in healthy individuals (20). Finally, because of robustness in the assay in terms of sensitivity, lability, and durability, we suggest that a pTyr-SH2 FRET cassette could serve as a general platform for technology to monitor drug resistance for other tyrosine kinases implicated in human cancer.

Materials and Methods

Materials, drugs, and antibodies
Imatinib mesylate was kindly provided by Novartis and dasatinib was from Bristol-Myers Squibb with appropriate Material Transfer Agreements. Immunoblotting was done using standard procedures with phosphorylated Crk II Y221 antibody (Cell Signaling Technology), and total c-Abl antibody (Calbiochem). Transfections were done with Fugene 6 (Roche) according to the instructions of the manufacturer.

Plasmids
The DNA for the Picchu FRET probe and for the membrane-targeted CAAX-tagged Picchu (Picchu-CAAX) have been previously described (19).

Cell culture and transfections
32D cells expressing WT Bcr-Abl and T315I Bcr-Abl were kindly provided by Dr. Michael Deininger (Oregon State Health Science Center). HEK 293T cells were obtained from the laboratory stock and CosE37 cells were provided by Dr. Michiyuki Matsuda. Authentication was not performed on 32D, 293T, and CosE37 cells by the authors. 293T cells were maintained in 1× DMEM supplemented with 10% fetal bovine serum and 2 mmol/L of L-glutamine in 37°C and 5% CO₂. Cells were plated onto either 6 cm or 12-well plates and, the next day, were transfected with 1 or 0.5 μg of bcr-abl. Twenty-four hours later, imatinib or dasatinib treatment was applied followed 2 hours later by transfection with 1 or 0.5 μg Picchu. Twenty-four to 48 hours later, cells were collected and FRET measurements were done on 50 to 60 μg of total protein. Western blot analysis was done on 15 to 20 μg total protein as described above. CosE37 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L of L-glutamine in 37°C and 5% CO₂. Cells were plated onto either 6 cm or 12-well plates and, the next day, were transfected with 1 or 0.5 μg of bcr-abl. Twenty-four hours later, cells were either analyzed via flow cytometry or real-time FRET microscopy.

Plate reader FRET assay
FRET emissions were measured using a 96-well Bio-Tek Synergy HT plate reader equipped with 440/30, 485/20,
and 528/20 nm filters. Excitation of the probe was done at 440 nm and FRET emissions were read at 528 nm.

**Flow cytometry**

Twenty-four hours following transfection, CosE37 cells were trypsinized and analyzed on a Flicyme-320 flow cytometer (Mitsui Engineering & Shipbuilding, Co.) equipped with a 408 nm laser diode, a 540/30 YFP filter, and a 494/41 CFP filter. Data was analyzed on the Flicyme Data Station (Mitsui Engineering & Shipbuilding). Where appropriate, cells were treated with 5 μmol/L of imatinib for 10 minutes in suspension prior to the fluorescence-activated cell sorting (FACS) analysis.

**Real-time FRET microscopy**

CosE37 medium was replaced with 2 mL of DMEM F-12 medium (Life Technologies) and overlaid with 1.5 mL of mineral oil. Real-time FRET microscopy was done on an IX81 inverted microscope (Olympus) equipped with a laser-based autofocusing system (ZDC), a 440AF21 excitation filter (Omega), a 455DRLP Dichroic mirror (Omega), a 535AF26 emission filter (Omega), an automatic XY stage controller (Sigma Koki), a 60×/1.4 oil immersion objective lens (PlanApo), and a CoolSNAP HQ CCD camera (Roper Scientific).

**Statistical analysis**

Analysis of a minimum of five cells is recommended when performing time-lapse FRET imaging (21). To attain a more accurate measurement of FRET emissions, however, we opted to select 13 to 16 cells. Although, due to the inherent variability among cells in culture, the varying efficiencies of transfection and gene expression, and the unequal exposure of the cells to administered therapeutic agents, the observed responses to tyrosine kinase inhibitor application differed from cell to cell. In this article, we show data from representative cells of each treatment group, with aggregate inhibition profiles available as supplemental data.

**FRET inhibition rate constants**

Rate constants for the inhibition of FRET following imatinib or dasatinib treatment were calculated as the decrease in FRET over a particular time interval divided by the length of the time interval ($r = \Delta \text{FRET}/\Delta T$)

**Image analysis**

Quantitation of immunoblots was done with GeneTools software (Syngene) whereas real-time FRET imaging was done using MetaMorph Software (Roper Scientific).

**Results**

**Application of Picchu FRET for analysis of drug resistance to Bcr-Abl in CML**

The FRET assay described here exploits the interaction between the Abl tyrosine kinase and the Crk II adaptor protein (22, 23). Following an interaction between Crk and Abl or Bcr-Abl, Crk II is phosphorylated on Tyr221 (Fig. 1), and therefore, adopts a closed conformation bringing the NH2- and COOH-terminal ends together (24), a conformation that has been validated by structural analysis (24, 25). The Picchu probe is similar to the Crk II protein with the addition of an NH2-terminal YFP (yellow...
mutant of green fluorescent protein) and a COOH-terminal CFP (cyan mutant of green fluorescent protein). However, unlike Crk II, Picchu lacks the SH3C following Tyr221 (19), which deletes most of the linker region and SH3C domain and therefore makes the Picchu fusion protein more compact, increasing the FRET efficiency following tyrosine phosphorylation (19). Because phosphorylation of Tyr221 in Picchu by Bcr-Abl also causes an SH2-\(p\)Tyr intramolecular interaction, allowing the molecule to adopt a closed conformation similar to Crk II (bringing the YFP and CFP within close enough proximity for FRET), we reckoned it is well-suited to serve as an \textit{in vivo} probe to analyze Bcr-Abl activity and inhibition in real-time (Fig. 1).

To verify that Crk Tyr221 phosphorylation is representative of Bcr-Abl activity, we analyzed Crk phosphorylation in Bcr-Abl–expressing 32D cells in the presence or absence of imatinib (Fig. 2A). Tyr221 was highly sensitive to imatinib in WT Bcr-Abl 32D cells, whereas 32D cells expressing a T315I imatinib-resistant Bcr-Abl mutant were largely insensitive (lane 2 versus lane 4 in Fig. 2A). Similar results were observed in HEK 293T cells expressing clinically relevant Bcr-Abl mutants Y253F, E255K, and T315I and transfected with Picchu (Fig. 2B). Patients with Bcr-Abl T315I mutants fail imatinib treatment because this substitution near the P-loop structure of the kinase domain blocks drug binding (4). Analogous to the case with Crk, Picchu phosphorylation mirrored the inhibition of the aforementioned mutants as well as the resistance to inhibition of T315I Bcr-Abl (Fig. 2B). To extrapolate this effect into a biological assay, a time course assay was done whereby Picchu and Bcr-Abl were coexpressed in 293T cells, after which, imatinib was added for 0.5, 1, 2, and 4 hours (Fig. 2C). As shown, Bcr-Abl was inhibited (dephosphorylated on Tyr245) in a time-dependent manner, which correlated well with Picchu Tyr221 dephosphorylation. Interestingly, both proteins are dephosphorylated at the earliest time point investigated (30 min). This suggests that the effects of TKIs are rapid, which is a prerequisite for the development of a real-time FRET assay for Bcr-Abl.

![Figure 3. A, effect of Bcr-Abl tyrosine kinase mutations on Picchu FRET. Phosphorylation of the Picchu probe by Bcr-Abl and the corresponding changes in FRET emissions after imatinib treatment. Columns, duplicate samples; only one sample is shown for the T315I mutant in this experiment. B, real-time FRET microscopy demonstrating pseudocolor visualization of the change in FRET emission after the addition of 5 \(\mu\)mol/L of imatinib for WT Bcr-Abl (i and ii) and the T315I mutant (iii and iv). See supplemental movie Supplementary Fig. S1 and Supplementary Fig. S2 for real-time kinetics as well as the accompanying graph, Supplementary Fig. S3, depicting FRET tracking for each cell in Supplementary Fig. S2. C and D, real-time microscopic “tracking” of FRET emissions in CosE37 cells expressing WT Bcr-Abl (C) and T315I Bcr-Abl (D). DMSO (as control) or imatinib was added at 19 min. Multi-cell FRET tracking can be found as supplemental data (Supplementary Fig. S4).]
activity. Indeed, as shown below, Picchu-FRET occurs within minutes after imatinib, suggesting that it is well suited for in vivo manipulation. Based on these facts, we posit that Picchu Tyr221 phosphorylation is highly labile to dephosphorylation by TKIs and acts as a surrogate marker for Bcr-Abl activity following TKI administration.

FRET emission from Picchu reflects activation status of Bcr-Abl and response towards TKIs

Having established that Bcr-Abl activity correlates with Picchu Tyr221 phosphorylation using immunoblot analysis, we analyzed 530 nm FRET emission from the Picchu probe using a Bio-Tek Synergy HT plate reader to observe if FRET correlated with phosphorylation. As shown in Fig. 3A, total Picchu FRET emission shared a consistent pattern of dephosphorylation after imatinib treatment. More importantly, when measured against the change in FRET emission with and without imatinib, we found that Bcr-Abl and those mutants responsive to imatinib showed a significantly decreased FRET (WT, Y253F, and E255K), whereas T315I showed resistance to inhibition in the FRET analysis (Fig. 3A), demonstrating the potential of Picchu-FRET assay to monitor the acquisition of drug resistance in vivo.

We next employed FRET microscopy to track FRET emissions in real-time (Fig. 3B–D). Application of imatinib resulted in a rapid and dose-dependent decrease in the FRET ratio (YFP/CFP) in CosE37 cells expressing WT Bcr-Abl (Fig. 3C, Supplementary Fig. S4), but not in CosE37 cells expressing T315I Bcr-Abl (Fig. 3D, Supplementary Fig. S4). Figure 3B shows the utility of Picchu FRET in real-time microscopy. Note that the addition of imatinib suppressed WT Bcr-Abl activity (i versus ii), but was resistant in cells expressing T315I Bcr-Abl (iii versus iv). Such an approach has the potential for application in rapid and sensitive screening of novel drug candidates that inhibit T315I Bcr-Abl in vivo (see supplemental movie for in vivo analysis, Supplementary Fig. S1, as well as dose-response data, Supplementary Fig. S2; also see Supplementary Fig. S3 for the tracking of FRET emissions for each cell in Supplementary Fig. S2).

Flow cytometric demonstration of FRET probe utility

To show the potential for using Picchu-FRET in analyzing large numbers of cells, for example, to monitor drug resistance in a mixed population clinical sample from bone marrow or peripheral blood, we did flow cytometric studies on CosE37 cells transfected with WT Bcr-Abl and either a membrane-targeted (Picchu-CAAX) or -untargeted (Picchu) FRET probe (Fig. 4). Although membrane localization of the probe seemed to produce a more specific response, perhaps due to less background as compared with the widely distributed Picchu probe, with either biosensor both the increased FRET ratio in active Bcr-Abl-expressing cells as well as the decrease in the ratio following imatinib treatment were rapidly detectable, suggesting it should be feasible to collect rare populations of drug-resistant cells prior to complete leukemic relapse.

Pharmacodynamic responses of Picchu FRET to imatinib and dasatinib

Having established the utility of Picchu as a bioprobe for imaging drug resistance in CML at both the light microscopic level and by FACS staining, we next wanted to evaluate if we could compare sensitivities to different TKIs used clinically, i.e., dasatinib, a 2-amino-thiazole-5-carboxamide, and imatinib. Dasatinib and imatinib have distinct bioavailability in vivo, with dasatinib being 300-fold more potent than imatinib in suppressing unmutated Bcr-Abl, at least partly owing to the fact that imatinib binds to the inactive configuration of Abl, blocking transition to the active configuration (26, 27), whereas dasatinib binds to both the active and inactive P-loop configurations (9). Interestingly, these sensitivity differences were clearly reflected in the responses of cells to TKIs in the Picchu FRET assay (Fig. 5). Hence, not only did FRET...
depression following Bcr-Abl inhibition by both imatinib and dasatinib display striking dose-dependency in vivo (μmol/L for imatinib and nmol/L for dasatinib), but the rate constants for inhibition were also characteristically different. This suggests that it is possible to identify beneficial synergistic interactions of TKIs with respect to both the level and the duration of kinase inhibition, thus taking advantage of the differences between various drugs in terms of bioavailability and mechanisms of inhibition. Naturally, the corollary to this notion is the utilization of this assay to identify compounds that should not be used in combination, by virtue of their competitive nature. Indeed, after the initial inhibition of Bcr-Abl and concomitant FRET depression following 3 nmol/L of dasatinib treatment in Fig. 6, the subsequent application of 4.2 μmol/L of imatinib results in an initial plateau.
followed by the resumption of kinase suppression, suggesting possible competition between the two compounds, either in binding to Bcr-Abl or in cellular uptake, thus allowing for a momentary reactivation of the kinase.

To further validate the Picchu FRET bioprobe as a potential pharmaceutical tool to be used in the development of next-generation TKIs, we constructed two examples of three-dimensional Bcr-Abl inhibition profiles (Fig. 7). This approach allows for the observation of the dynamic inhibition of Bcr-Abl in a time-dependent manner following administration of an array of kinase inhibitors in various concentrations. Such a profile can be created for the inhibition of each clinically relevant Bcr-Abl mutant by every currently available TKI providing the unprecedented ability to rapidly identify the Bcr-Abl mutant(s) driving a patient’s leukemia and administer the kinase inhibitor, or combination of inhibitors, that will push the patient into remission with the greatest potency, longest duration, lowest drug concentration, and fewest side effects. In this manner, the treatment of CML may be converted from the current “one size fits all” modality to a significantly more tailored management of the patient.

Discussion

The advent of imatinib mesylate over a decade ago was a breakthrough in cancer therapy and resulted in a paradigm shift with respect to molecular targeting of oncogenes. Its remarkable success in clinical trials resulted in fast-track approval by the Food and Drug Administration, and imatinib continues to be a frontline therapy today (11). Despite the fact that 5-year survival for patients on imatinib is currently 95%, patient relapse and drug resistance continues to make the treatment of CML difficult (12, 28). Often, individuals who have become refractory to a certain TKI are switched to another and although this approach benefits some patients in the short-term, it is not a complete cure, and in particular, those individuals harboring mutations in critical residues such as T315 are untreatable by any means currently available. However, recent investigations have identified potential combinations of kinase inhibitors not only in the case of T315I Bcr-Abl, but also with regard to the resistance-inducing efflux of therapeutic compounds by the MDR1 transporter (29, 30). Such findings give credence to the potential for identifying synergistic combinations of therapeutic agents and the utility of the described FRET assay in rapidly and sensitively making such determinations.

The frequent occurrence of drug resistance in oncogene-targeted therapy, in combination with the development of robust second-generation TKI inhibitors, underscores the importance for rapid and selective screening of drug resistance in vivo. Moreover, whereas Bcr-Abl inhibitors initially induce moderate-term remission, it would be of clinical value to begin second-line TKI therapeutics when resistance first emerges in a few repopulating cells. Because the Picchu FRET assay described here can be adapted for FACS analysis and FACS sorting through a combination of surface marker labeling and detection of Bcr-Abl activity in vivo, the assay can be used to identify rare leukemic cell lines, and to indicate their inhibition by or resistance to the applied drug therapy. Such an assay could conceivably be used to analyze patient samples to diagnose CML, confirm Bcr-Abl activity after a diagnosis of CML is suspected, detect relapse in CML patients already receiving treatment, or to determine the therapeutic or combination of therapeutics that would most benefit the patient after their Bcr-Abl mutation has been identified. In this regard, a FRET assay has the added benefit of not only identifying Bcr-Abl but in providing information on its activity—in contrast with currently available diagnostic tools, such as nested PCR, which, although sensitive, simply identify the Bcr-Abl fusion oncogene but provide no useful information with regard to its activation status, a significant shortcoming considering the oncogene has been detected at low levels in healthy individuals (20).

A second utility of Picchu FRET arises from its strong potential as a highly effective pharmaceutical tool that...

![Image](328x196 to 528x470)

**Figure 7.** Pharmaceutical profiling of Bcr-Abl inhibition by TKIs. Two examples of the potential utility of Picchu FRET in pharmaceutical profiling of clinically relevant Bcr-Abl mutations as to their efficient inhibition by therapeutics of varying concentrations. Inhibition is depicted as a function of TKI concentration (left), time (bottom), and ΔFRET ratios (right) for imatinib (top) and dasatinib (bottom) to provide a pharmaceutical signature for Bcr-Abl proteins. These data can be extrapolated to select the optimum therapeutic, or combination thereof, for different Bcr-Abl mutations for the selection of TKIs with the most desirable kinase inhibition profiles.
can be used to rapidly screen and identify novel small molecules able to inhibit Bcr-Abl in vivo at lower doses and even candidates that are able to inhibit T315I Bcr-Abl and other such detrimental mutations. Furthermore, this assay may also lend itself to application in the determination of the cell type specificity of a particular CML case through dual labeling for cell surface markers, which may lead to more targeted treatments based on the lineage of the leukemic cells in question. Additionally, the distinct differences in the response of WT Bcr-Abl to imatinib and dasatinib as measured by the FRET emissions suggests that Picchu FRET may also be able to reflect the inherent pharmacokinetic variations between therapeutics based on variables such as mechanism of action, for example, dual Src/Ab1 kinase inhibition by dasatinib (31), and their differing uptake and efflux profiles. Although numerous uses can be developed for such an assay, it should be stressed that the sensitive and, perhaps more important, in vivo nature of this assay creates certain possibilities and provides a degree of utility that has not been realized with other techniques used to date.

By examining changes in FRET ratios as a function of imatinib concentration, we estimate that the half-life of Tyr221 phosphorylation was ∼3 minutes following Bcr-Abl inhibition. This is quite interesting at a mechanistic level because it predicts the existence of a potent Tyr221 phosphatase that could be identified following a genome-wide short hairpin RNA screen against tyrosine phosphatases. As such, we predict that knockdown of a Crk PTPase would significantly delay or prevent the change in FRET ratio. Finally, based on the promising preliminary results of the Picchu probe, we propose that such a strategy may have a more generic utility to assess additional tyrosine kinases and their accompanying drug resistance in human cancer. Therefore, it should be feasible to adopt a general strategy to develop a platform of FRET probes that extrapolate the basic paradigm for Picchu. Specifically, the backbone of Picchu would be modified with two cassettes in place of (a) pTyr221 and (b) the Crk SH2 domain. Accordingly, for each tyrosine kinase to be examined, an optimal phosphorylation motif could be inserted in place of pTyr221 (cassette no. 2), and an optimal SH2 that associates with the pTyr could be inserted in place of the Crk SH2 domain (cassette no. 1; Fig. 8). As an example, the PVPEYIQSVKPRK peptide found in the COOH terminus of epidermal growth factor receptor containing tyrosine 1068 that is autophosphorylated by the kinase upon epidermal growth factor stimulation (32) could be substituted for the Tyr221 region of Picchu. Likewise, substitution of the original SH2 domain of Picchu by the SH2 domain of Grb2, which binds to Tyr1068 of epidermal growth factor receptor upon its phosphorylation (32), could conceivably produce a dynamic probe for the real-time activity and inhibition of the epidermal growth factor receptor kinase, which has been implicated in numerous cancers.

With the recent advancements in rapid profiling of disease states both at the genomic and proteomic levels coupled with the prospective widespread genetic blue-printing of patients and their risk factors, modern medicine is undergoing a paradigm shift from the conventional generalized approach of treatment based on stratification by disease states, to a more custom-fitted and individual-centered modus operandi. In the same vein, with the introduction of oncogene-directed cancer therapy by the
advent of imatinib mesylate, and the extensive list of resistance-conferring Bcr-Abl kinase mutations, we are beginning to realize the possibility of tailor-made therapeutics in the treatment of CML. The real-time FRET assay, as presented in this report, may be an invaluable factor during this transition and through its versatility and multi-faceted utility as both a clinical tool and as an instrument of rapid real-time therapeutic development, could provide the necessary technology to push through the event horizon into an age of patient-centered medicine.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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