Emerging applications of kinetic biomarkers in preclinical and clinical drug development

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The cost of drug discovery and development is increasing, while the rate of new drug approvals is declining. In contrast to major technological advances with in silico and in vitro screening tools, there have been almost no advances in the tools available for establishing the actions of agents in the complex biochemical networks characteristic of fully assembled living systems. The resulting poor capacity to predict clinical response underlies the high attrition rate of leads at every step of drug development. A potential solution would be provided by kinetic biomarkers (in vivo measurement of fluxes though the key pathways that drive disease processes and therapeutic response). Novel approaches using stable isotope labeling with mass spectrometric analysis have recently emerged for measuring molecular kinetics relevant to drug targets with some applications to drug development. This review discusses the general principles of kinetic biomarkers, providing examples where kinetics have generated meaningful insights into drug activity and highlighting areas where the application of kinetic biomarkers may be particularly useful for future drug discovery and development. Stable isotope mass spectrometric technologies may provide a parallel efficiency for converting molecules into approved drugs with sufficient throughput and reproducibility to maintain pace with the modern engine for generating leads.

Keywords Drug discovery and development, high-throughput screening, kinetic biomarker, mass spectrometry, stable isotope

Introduction

Contrary to the expectations engendered by modern technologies for identifying drug targets and leads, the rate of new drug approvals has not accelerated, but has slowed dramatically in recent years [1•,2]. The disparity between the increased generation of molecular leads, or new chemical entities (NCEs), through *ex vivo* high-throughput screening (HTS) assays, and the approval of safe, effective drugs, requires explanation.

The Achilles heel of drug discovery and development

Attrition, or high failure rate, has emerged as a central problem of the modern drug discovery and development (DDD) system [3•]. This should not be surprising, since modern DDD has focused largely on molecules, rather than their actions in living systems, but such molecules are not necessarily drugs. *Ex vivo* assays, no matter how elegant, cannot predict the clinical response to an agent in a complex, living organism, particularly a human being with a disease condition. As highlighted in a recent Food and Drug Administration (FDA) white paper, 'development science' has failed to keep up with 'discovery science' [1•]. On the molecular discovery side, great advances have been made in target identification and lead optimization, including, for example, the development of X-ray crystal structures of targets and powerful medicinal chemistry tools. In contrast, nearly every aspect of DDD following the creation and elucidation of a molecule (development science) is almost the same as it was in the 1950s. The missing link in modern DDD is therefore a comparable efficiency for the conversion of NCEs to approved drugs as exists for the creation of NCEs, with sufficient throughput and reproducibility to keep up with the modern engine for generating molecular leads.

When viewed from a broad perspective, the modern DDD paradigm has created an untenable system. The combination of enormously efficient tools for identifying leads, particularly those active against biologically novel or 'unvalidated' targets, with no equally efficient process for filtering, eliminating or optimizing these leads on the basis of their actions in living organisms, has resulted in a clogged pipeline. The current system is, unfortunately, perfectly designed for the exorbitant cost per drug approved that characterizes this era [2].

High attrition rates: Poor predictive power

A probable cause of high attrition is a poor predictive capacity for clinical response [1•,3•]. Pharmaceutical researchers are unable to predict the likely success or failure of agents using the tools available. An inability to link molecular events (ie, actions on the physical targets of drugs) to functional outcomes (ie, macroscopic events that beneficially alter disease processes without causing undesired toxicities) is responsible for the extremely low success rates of leads that are prosecuted in modern DDD.

This situation can be improved. Comparison to the 'golden era' of drug discovery (1940s to 1960s) is instructive. This was the era in which most of the noteworthy drugs of modern man were discovered (eg, antibiotics, glucocorticoids and antipsychotic agents). Drug discovery in this era was based almost exclusively on macroscopic functional outcomes and trial and error, ie, *in vivo* experimentation [4] with little basic understanding of the molecular targets or mechanisms involved. Although this was an inefficient system for generating leads - and indeed the pipeline ran dry - once a candidate was identified, the

likelihood of clinical success was higher [4]. Macroscopic phenotypic outcomes were low-thoughput and imprecise, but more highly predictive than *ex vivo* HTS measures.

Is there a solution?

There exists, in principle, a solution to this imbalance between throughput and predictability. The ability to objectively measure the biochemical actions of agents on their true targets in living systems would provide an objective means of establishing efficacy and predicting clinical response. The true functional biochemical targets of drugs are fluxes of molecules through the pathways that are responsible for disease in fully assembled systems. Metrics of disease processes or therapeutic activity are termed biomarkers. 'Authentic biomarkers' reveal the activity of a pathway that is integrally involved in disease activity or therapeutic action. The absence of authentic biomarkers is arguably the key to the lag in drug development [1•] and is the major current impediment to advancing molecules to drugs.

For a biomarker to be authentic, it must possess two features. Firstly, it must distill the essence of a complex, emergent and functionally significant process into a precise physical-chemical measurement, and secondly, it must have a causal role and be centrally involved in the activity, severity, progression or reversal of the disease such that dissociation from clinical outcome is unlikely. Also, to be useful in modern DDD, such biomarkers must exhibit sufficient throughput and reproducibility to be able to meet the demands of the high levels of lead generation through HTS assays.

Stable isotopes have been used since the 1930s to study the kinetics of carbohydrate, lipid and protein metabolism in man, revealing the fundamental features of human metabolism [5,6,7•]. Classically restricted to academic study, new approaches and applications of stable isotopes and mass spectrometry (MS) are now being developed for, and applied to the DDD arena. The remainder of this review will focus on stable isotope-mass spectrometric kinetic biomarkers and their uses in DDD.

Kinetic biomarkers - advantages over static measures

Kinetic biomarkers have several intrinsic advantages over static markers of disease, as has been extensively reviewed elsewhere [8,9••,10,11•]. One extremely useful feature derives from the fact that changes in flux always precede changes in pool size or content, and are typically of greater magnitude than changes in pool size. For example, consider the testing of an inhibitor of collagen synthesis in a model of liver fibrosis. Over a period of 2 weeks, the liver collagen content increases from 100 to 106 units, a modest but significant rise. The inhibitor reduces this increase to 103 units. Although comparison of the collagen content is unlikely to detect the $< 3\%$ difference (106 versus 103), the measurement of flux (kinetics) would reveal a difference of 50% (3 versus 6), a magnitude that is easily detectable and significant with a relatively small sample size.

While stable isotopes and kinetic measurements are commonplace in physiology and metabolic studies, there are some useful distinctions between the application of kinetics to DDD and to physiology research. For example, a metabolic researcher may be interested in the percent contribution of gluconeogenesis to plasma glucose in diabetes, whereas the application of a kinetic biomarker for drug development may only need to detect whether a drug lowered gluconeogensis and by how much. Another distinction relates to the non-steady state. Most physiological studies are performed under steady state conditions, however, therapeutic interventions often induce a non-steady state. Measures of label incorporation in the non-steady state are ideally suited to kinetic biomarkers, because simple changes in label incorporation characterize non-steady-state conditions independent of mathematical models.

Kinetic biomarkers - limitations

Kinetic biomarkers possess some intrinsic limitations compared with other types of biomarkers. By their nature, kinetic tests cannot be performed post hoc, and therefore cannot be applied to banked tissue or archival samples. Currently, kinetic biomarkers are not well suited to measuring intracellular signaling processes (phosphorylation, sulfation), as opposed to pathways catalyzing molecular fluxes, although the effects of modulating intracellular signaling processes are amenable to the technology. The sensitivity of current MS also limits some applications of kinetic biomarkers, since sufficient (picogram) amounts of material from the molecule of interest must be isolated for analysis.

Measuring molecular flux rates in living systems

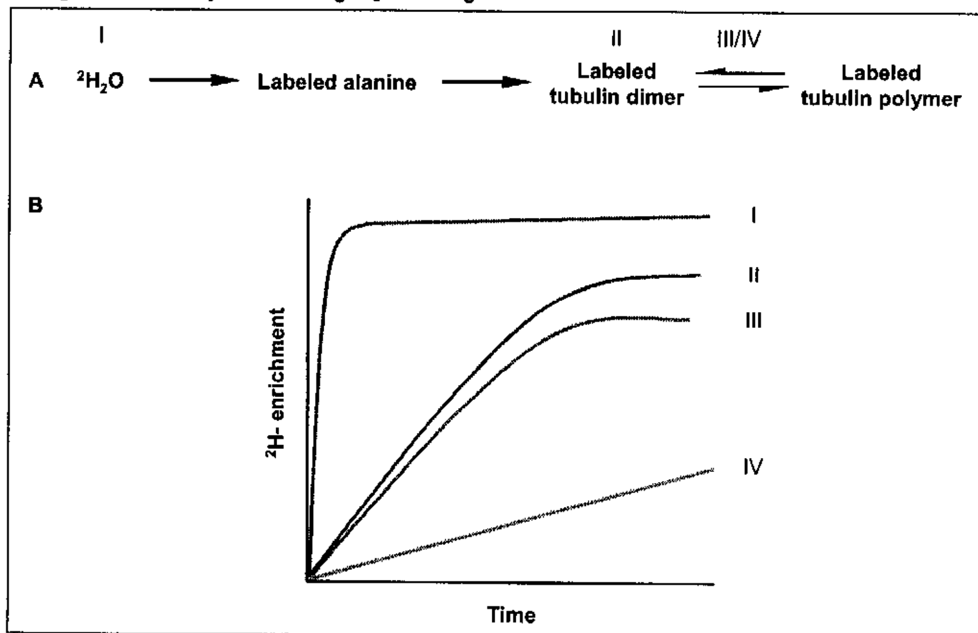
The following sections focus on two broad categories of kinetic biomarkers, one in which a specific cellular pathway is measured (eg, microtubule polymerization, ribonucleotide reductase) and the other in which the general property of the system is observed (eg, cell proliferation). These measurements can typically be made simultaneously, offering even greater insight into the regulation of the system.

Cancer biology

Microtubule dynamics

Drugs targeting microtubule dynamics are among the most important cancer chemotherapeutic agents (eg, taxanes), although in the past there was no direct way of determining the activity of agents that alter microtubule polymerization or depolymerization. The dynamic equilibrium between tubulin dimers and tubulin polymer can be measured by introducing a label into the free dimer pool, for example, in the form of ^2H_2O labeling of non-essential amino acids entering free tubulin dimers (Figure 1). Fanara *et al* recently used this ^2H_2O labeling approach to quantify the effects of taxanes on microtubule dynamics and cell proliferation in experimental tumors [12•]. In the absence of taxanes, equilibration of the label is observed between free dimers and total polymer (Figure 2A). Paclitaxel exposure results in a labeling gradient between dimer and polymer in cultured cells (Figure 2B) and implanted tumors (Figure 3), reflecting inhibition of microtubule dynamics. The degree of label gradient correlates with the inhibition of cell division (Figure 4). Thus, microtubule dynamics can be measured using ^2H_2O labeling and may provide a biomarker of taxane activity *in vivo*, including in humans with cancer.

Figure 1. Measuring microtubule dynamics using ^2H_2O labeling.

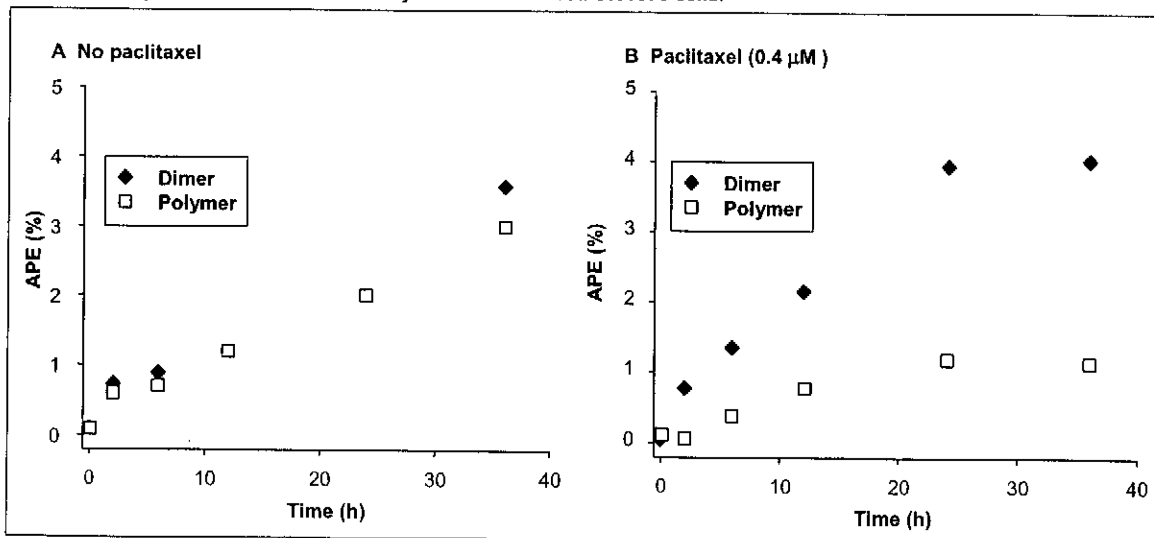


(A) ^2H atoms from heavy water (^2H_2O) are incorporated into the C-H bonds of non-essential amino acids such as alanine. The ^2H label is then incorporated into newly synthesized tubulin dimers, which in turn are incorporated into microtubules. (B) When microtubules are dynamically unstable, there is rapid exchange between dimers and polymers, and the ^2H label accumulates in polymers at almost the same rate as it appears in dimers. In contrast, when microtubules are less dynamic, or when dynamic exchange with tubulin dimers is disrupted by the action of microtubule-targeted, tubulin-polymerizing agents (MTPAs), ^2H enters the dimer pool at the biosynthetic rate, whereas incorporation into polymers is diminished.

I ^2H_2O, **II** dimer, **III** polymer in dynamic equilibrium with dimer, **IV** polymer in slow equilibrium with dimer or with MTPA present.

This figure is based on reference [12•].

Figure 2. Effect of paclitaxel on microtubule dynamics in cultured SW1573 cells.

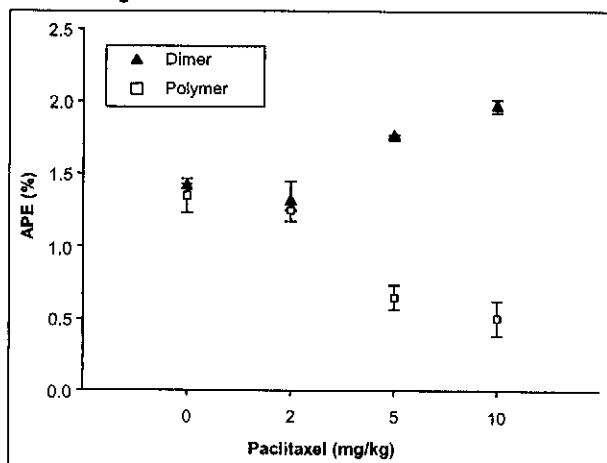


(A) SW1573 human lung cancer cells were labeled with ^2H_2O for 2, 6, 12, 24 and 36 h prior to harvest. Tubulin dimer and polymer fractions were purified from post-nuclear supernatants and ^2H label incorporation into tubulin-derived alanine was measured by gas chromatography (GC)/MS analysis. Tubulin dimers and polymers were in equilibrium on a time-scale of hours, reflecting rapid exchange kinetics between the two pools. (B) In contrast, in the presence of 0.4 µM paclitaxel, the rate of ^2H incorporation into polymers was reduced by 75%, compared to the rate of label incorporation into the dimer.

APE Atom percent excess of [^2H]alanine (^2H enrichment).

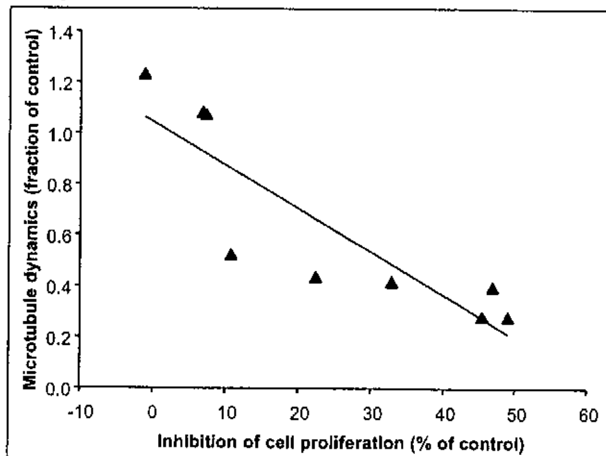
This figure is based on reference [12•].

Figure 3. Effect of paclitaxel dose on microtubule dynamics in tumor xenografts.



Tubulin dimer and polymer fractions were purified from SW1573 tumors in nude mice labeled with ^2H_2O for 24 h. [^{2}H]APE ^{2}H incorporation) in alanine was quantified by GC/MS (mean ± standard deviation for groups of three mice). In untreated mice, ^{2}H-label incorporation into tubulin dimers and microtubule polymers was indistinguishable, reflecting rapid exchange between the two pools (dynamic equilibrium). In contrast, paclitaxel treatment caused inhibition of ^{2}H-tubulin dimer incorporation into polymers, resulting in a dose-dependent inhibition of labeling in the polymer fraction.
This figure is based on reference [12•].

Figure 4. Relationship between the effects of paclitaxel on microtubule dynamics and cell DNA turnover in tumor xenografts.



SW1573 tumor tissue from xenografted, 24-h ^2H_2O-labeled, paclitaxel-treated mice was analyzed for ^{2}H-label incorporation into tubulin dimers and polymers (compare with Figure 3), and an index of microtubule dynamics was calculated as the ratio of ^{2}H enrichment in microtubules to that in free dimers from the same tissue sample (approximately one in untreated tissue). DNA was extracted from aliquots of tumor tissue, and used to quantify ^{2}H incorporation into deoxyribose moieties of purine deoxyribonucleotides by GC/MS (a measure of *de novo* DNA synthesis). Inhibition of DNA synthesis was calculated as the percentage decrease in ^{2}H incorporation in paclitaxel-treated, compared to untreated tumors (X-axis), and correlated with the index of microtubule dynamics (Y-axis).
This figure is based on reference [12•].

Hypomethylation

Hypomethylating agents, such as decitabine (5-aza-2'-deoxycytidine) and 5-azacytidine, target the methylation of deoxycytidine (dC) in DNA [13]. Davis *et al* have recently published elegant studies of folate-dependent methylation of homocystine and the flow of isotopically labeled serine and methionine in cells and in humans [14]. From the data produced, it is evident that *S*-adenosylmethionine (the methyl donor for DNA) can be labeled sufficiently *in vivo* with either tracer. The effect of DNA methylation inhibitors on the incorporation of ^{2}H from $^2H_3$-methionine into methyldeoxycytidine has been examined in cell culture (Figure 5), and it is clear that both decitabine and 5-azacytidine interfere with DNA methylation. This study, together with the results of Davis *et al* from humans, demonstrates that quantitative determination of the rate of DNA methylation and the effect of hypomethylating agents *in vivo* is now possible.

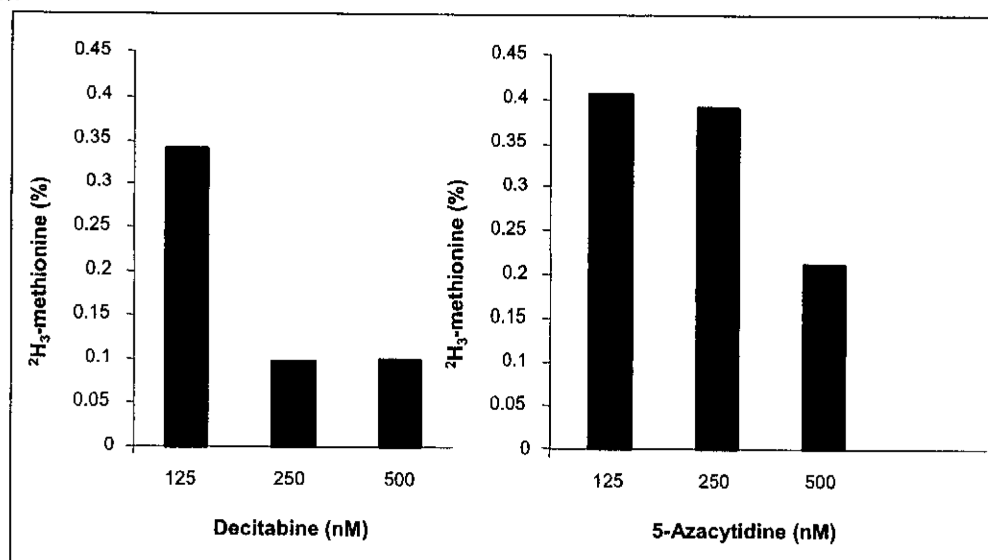
Ribonucleotide reductase

Ribonucleotide reductase (RR) inhibitors, including hydroxyurea (HU) and gemcitabine (gem), have a long history in cancer therapeutics [15]. No simple method has previously existed, however, for measuring flux through RR into deoxyribonucleotides. Awada *et al* described a method where the increase in pyrimidine deoxynucleotide (dN) salvage relative to purine dN salvage was used as a biomarker of RR activity [16]. In practical terms, the *de novo* synthesis of deoxyadenine (dA; constitutive) is compared with the *de novo* synthesis of deoxythymine (dT; regulated). Reduced *de novo* dT contribution to DNA relative to dA is a biomarker of inhibition of RR flux. The measurement was performed with a single isotopic tracer (^2H_2O), which was also used to simultaneously determine cell proliferation rates (see below). *In vitro* (Figure 6) and *in vivo* data (Figure 7) clearly demonstrate a robust dose response of the biomarker to RR inhibition in different cell types with both HU and gem. Furthermore, different tissue sensitivities to RR inhibition can be simultaneously observed, allowing the therapeutic index of a drug to be measured (Figure 8).

Metabolic profiling

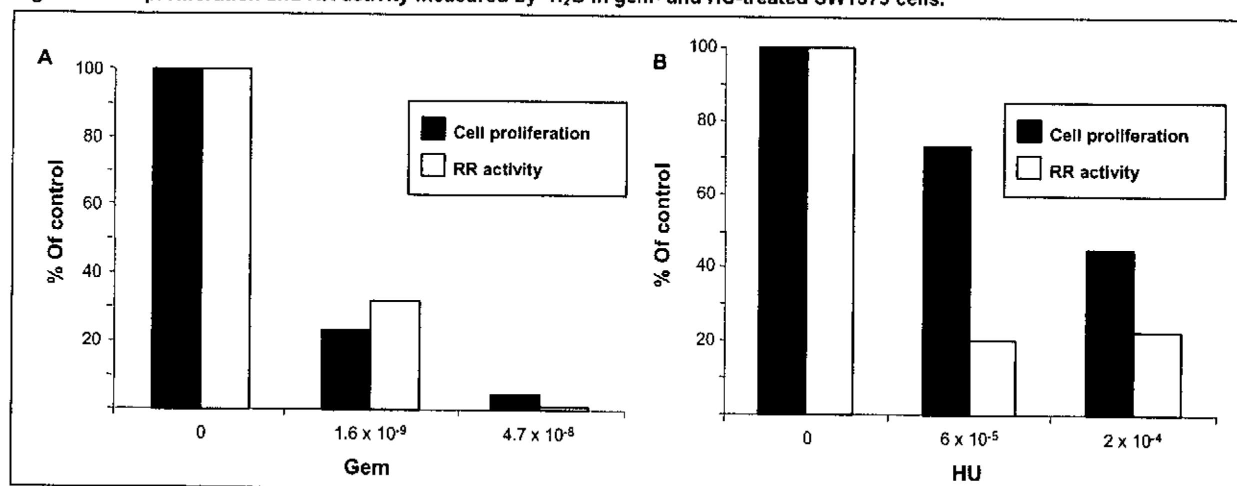
Lee *et al* described a novel approach to the metabolic profiling of tumor cells using 1,2-[^{13}C]-labeled glucose [17•,18]. A 'proliferative phenotype' of cancer cells can be assessed by distinguishing the fate of the ^{13}C label. The pattern or position of ^{13}C atoms on various glucose metabolites is determined by GC/MS, and changes in flux through, for example, glycolysis, the pentose cycle and lipogenesis, can be assessed from analysis of the respective down-stream metabolites (lactate, ribose, palmitate, CO_2, etc). Increased activity of the pentose phosphate pathway is indicative of poorly differentiated, long-lived, proliferating cells, whereas reduced carbon flow though the pentose pathway is associated with apoptosis in rapidly proliferating or dividing cells. Although apparently limited to *in vitro* applications, this approach provides detailed mapping of fluxes through critical pathways, which are modulated by chemotherapies. The combination of this approach with metabolic control analysis may identify new potent drug targets. Translation into *in vivo* models will increase the utility and importance of this approach.

Figure 5. Enrichment in methyldeoxycytidine from $^2H_3$-methionine in SW1573 lung cancer cells treated with decitabine and 5-azacytidine.



Cells were grown under standard conditions in media supplemented with $^2H_3$-methionine, and were harvested 24 h after incubation with agents.

Figure 6. Cell proliferation and RR activity measured by ^2H_2O in gem- and HU-treated SW1573 cells.



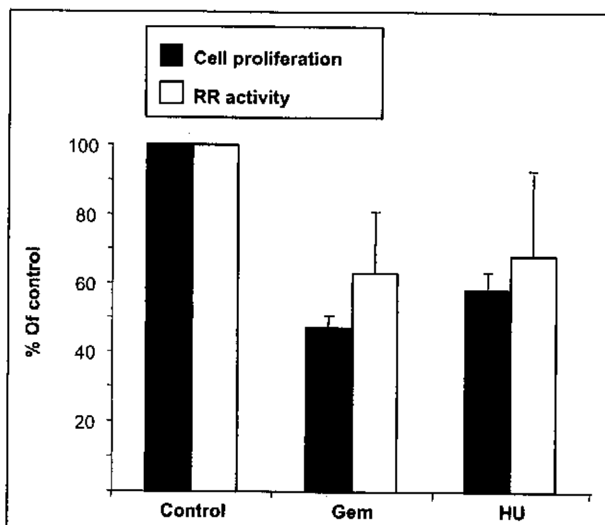
Non-small-cell lung carcinoma cells (SW1573) were grown under standard conditions to approximately 50% confluence. Media was removed and replaced with media containing 4% ^2H_2O, 1 μM dNs and HU (A) or gem (B) at various concentrations. Cells were grown for an additional 24 h and then harvested and frozen for GC/MS analysis.

Cell proliferation

Altered cell proliferation is an integral component of the carcinogenic process [19,20], as well as the immediate target of most cancer chemotherapeutic drugs. The capacity to measure cell proliferation rates in pre-cancer cells (prevention) or established cancer cells (therapy) would therefore provide a target or biomarker for *in vivo* intervention.

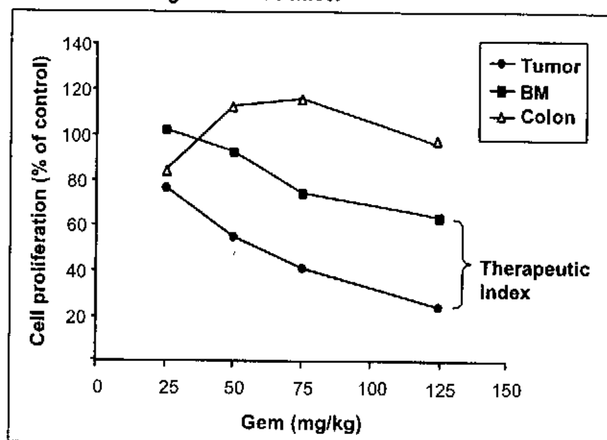
Misell *et al* used the ^2H_2O labeling technique (Figure 9) [21] to measure mammary epithelial cell (MEC) proliferation in response to selective estrogen receptor modulators (SERMs; Figure 10). Several conclusions can be drawn from these data. Firstly, raloxifene and tamoxifen exhibit dose-dependent suppression of MEC proliferation in intact female rats. Secondly, this inhibition also occurs in ovariectomized rats receiving exogenous estrogen, so the effect must be direct, not merely on the hypothalamic-pituitary axis. Thirdly, there is no agonist effect apparent even in ovariectomized animals. Finally, a relatively weak SERM (eg, genistein, a soy-derived compound) exhibited considerably less effect on MEC proliferation than raloxifene or tamoxifen. Thus, this kinetic approach is amenable to screening and comparing antiproliferative agents for breast cancer chemoprevention.

Figure 7. *In vivo* cell proliferation and RR activity in gem- and HU-treated mice.



Female BALB/c mice were implanted subcutaneously with approximately 10^6 mouse mammary carcinoma cells (EMT7) in Matrigel. Tumors were allowed to reach ~ 1500 mm^3 in size. Mice were then labeled with ^2H_2O and treated with either 125 mg/kg of gem or 500 mg/kg of HU. Gem was administered every other day, while HU was administered daily. At the end of 5 days, tumors were removed and RR activity and cell proliferation were measured.

Figure 8. Cell proliferation dose-response of tumor, colon and bone marrow to gem in nude mice.



Male BALB/c *nu/nu* mice were implanted subcutaneously with non-small-cell lung carcinoma cells (SW1573) in Matrigel. Mice were labeled with ^2H_2O and treated with increasing doses of gem, administered every other day. After 5 days, tumor, colon and bone marrow (BM) were removed and RR activity and cell proliferation were measured.

MEC proliferation was also measured using percutaneous biopsies from women (Figure 11) [22•]. A clear distinction between MEC proliferation in post-menopausal compared with pre-menopausal women can be seen, illustrating the translatability of the methodology from rodents to humans [22•].

Hsieh *et al* recently published data demonstrating the responsiveness of epidermal cell proliferation to carcinogens and antiproliferative agents [23••]. Their research presents the development of epidermal cell proliferation as a biomarker for chemoprevention. Beginning with a healthy turnover of epidermal cells *in vivo* the authors demonstrated both the incorporation and decay of isotopes in epidermal cells. From kinetics in healthy skin, an optimum labeling interval of 2 weeks was determined. 9,10-Dimethylbenz[a]anthracene (DMBA)/ 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were then administered both with and without lunasin, a putative antiproliferative agent. The results indicated a modest but significant effect of lunasin on both carcinogen-treated and healthy epidermal cell proliferation rates (Figure 12). Changes in the epidermal cell turnover rate of < 20% were statistically significant with only six animals per group, illustrating the analytical power of this approach.

Neurobiology

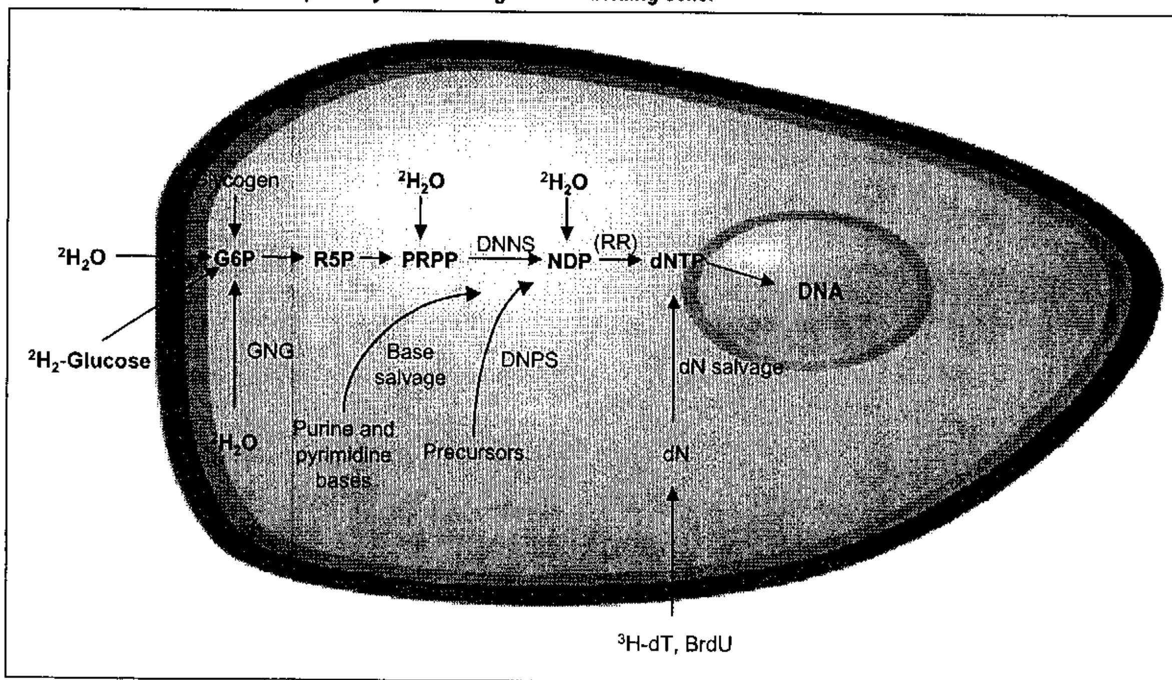
Although not a new approach, the application of bromodeoxyuridine (BrdU) labeling for the measurement of neurogenesis has recently become an active area of research. Of particular relevance to drug development is the observation that hippocampal neurogenesis appears to be a critical component in the activity of all known antidepressant agents [24]. Although the precise role of neurogenesis in the etiology of depression is uncertain [25] measurement of neurogenesis is clearly an attractive biomarker for antidepressant drug development. Furthermore, neurogenesis has been implicated in other neurological disorders such as Alzheimer's disease [26], schizophrenia [27] and learning disorders [25], suggesting that this may be a pharmacological target for many indications. Unfortunately, BrdU labeling has several intrinsic technical limitations, which prevent it from being an efficient tool for screening many compounds simultaneously. In particular, the time, imprecision and expense of staining and reading histological sections from an entire brain make this a low-throughput approach. It should be recognized that *ex vivo* techniques are of little value here (ie, neurogenesis must be measured in the intact brain).

Recently, Shankaran *et al* presented an alternative method for measuring neurogenesis using ^2H_2O [28]. The approach offers several advantages over BrdU labeling, such as improved quantitation, reduced labor and higher throughput. The effect of imipramine or fluoxetine to increase hippocampal cell proliferation was demonstrated using total hippocampal DNA isolation. In a separate study, mature neurons or neuronal progenitor cells were isolated by flow cytometry and the normal proliferation rate was shown to be increased by fluoxetine. These results suggest that ^2H_2O labeling is a viable approach for evaluating neuronal cell proliferation and neurogenesis *in vivo* in the living brain.

Metabolic diseases

Metabolic diseases such as obesity, insulin resistance and dyslipidemia, are emerging as dominant causes of morbidity and mortality worldwide. Consequently, the demand for effective pharmacological interventions is increasing. The complex, integrative nature of these diseases makes *in vitro* models less informative than for other diseases.

Figure 9. Schematic illustration of pathways for labeling DNA in dividing cells.



G Glucose, **PRPP** phosphoribosyl pyrophosphate, **GNG** gluconeogenesis, **P** phosphate, **R** ribose, **DNPS** *de novo* purine synthesis pathway, **DNNS** *de novo* nucleotide synthesis pathway, **NDP** nucleoside diphosphate, **RR** ribonucleoside reductase, **dNTP** deoxyribonucleoside triphosphate, **BrdU** bromodeoxyuridine.
This figure is based on reference [21].

Figure 10. Dose response of rat MEC to extremely low levels of SERMs in intact and ovariectomized rats.

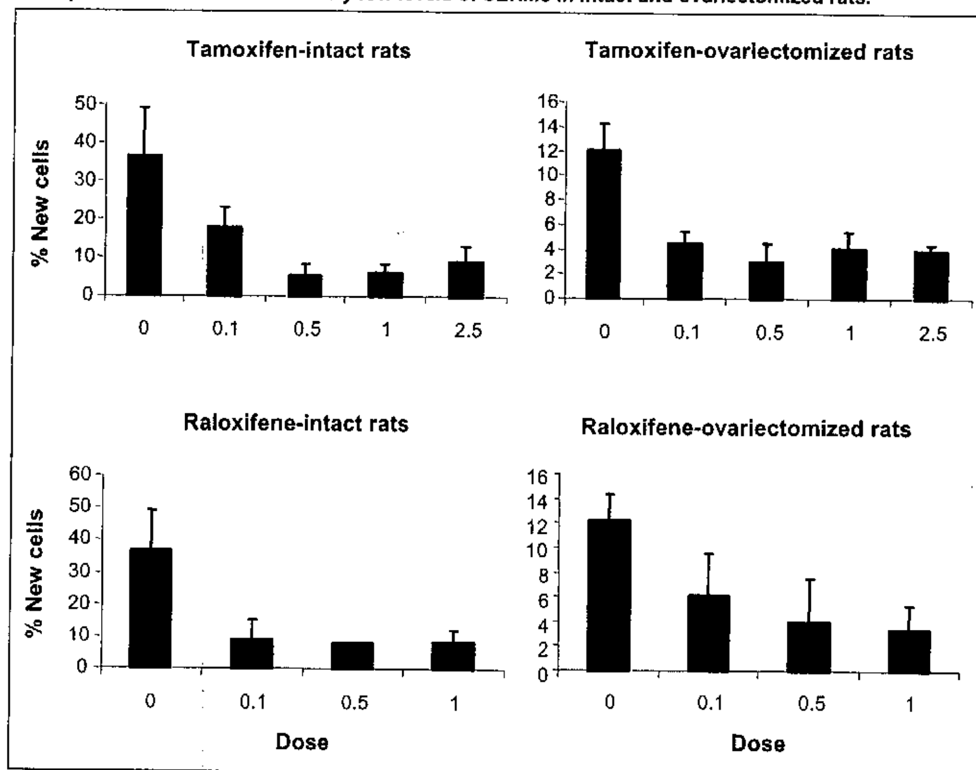
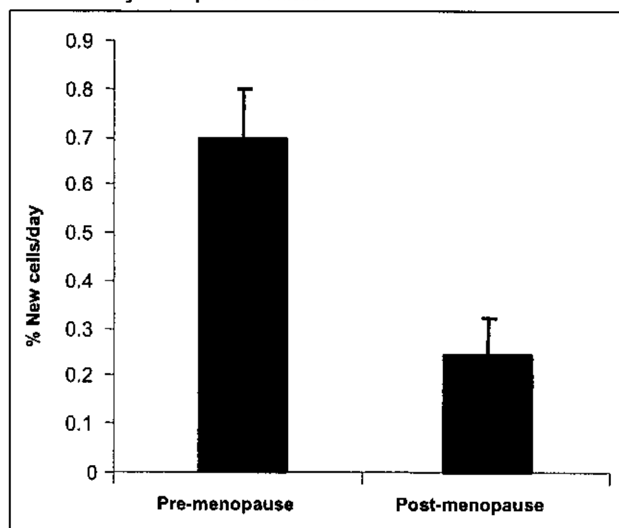
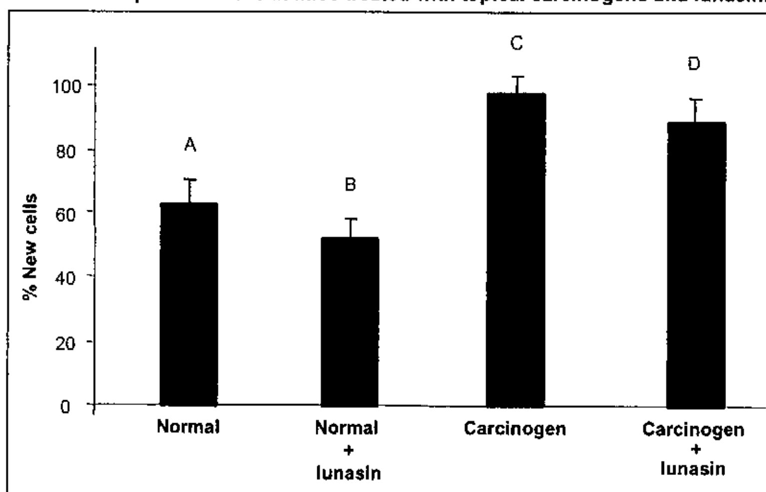


Figure 11. MEC proliferation rates stratified by menopausal status.



Post-menopausal women (n = 7) had significantly lower proliferation rates than pre-menopausal women (n = 18).
This figure is based on reference [22•].

Figure 12. Fractional replacement of epidermal cells in mice treated with topical carcinogens and lunasin.



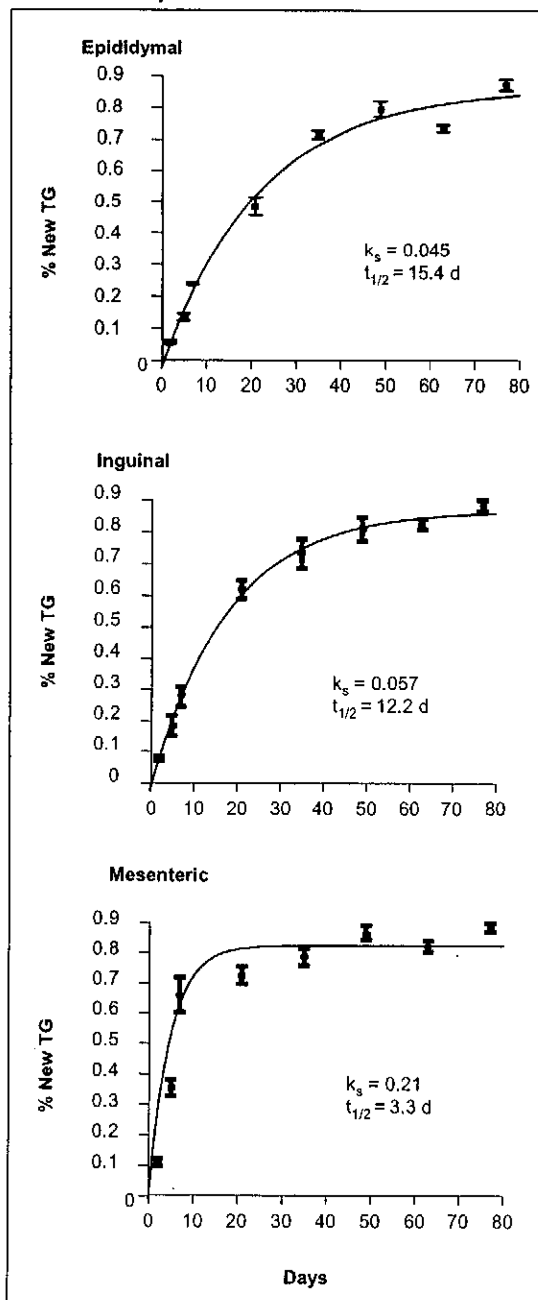
SENCAR mice treated with topical carcinogens DMBA and TPA were administered lunasin (D) or vehicle (C). Another group of SENCAR mice were given vehicle instead of the carcinogens and were also treated with topical lunasin (B) or vehicle (A). ^2H_2O label was administered during 2 weeks prior to euthanasia. Data are mean standard deviation (n = 6 per group). All of the groups demonstrated results which were statistically significantly different ($p < 0.05$) by one-way ANOVA with Bonferroni follow-up.
This figure is based on reference [23•].

Obesity and adipose metabolism

A new method for measuring adipose triglyceride (TG) kinetics *in vivo* using ^2H_2O was recently described [29••,30•]. The underlying principle comprises incorporation of ^2H into TG-bound glycerol. Previs *et al* evaluated the effect of diet composition on the rate of accumulation and turnover of adipose tissue TG, demonstrating that increasing the TG content of the diet from 10 to 45% fat resulted in the adipose TG turnover rate and net accumulation increasing by over 2-fold [29••]. Turner *et al* described dramatic differences in the turnover of TG in mesenteric compared to peripheral adipose depots (Figure 13), confirming a long-held hypothesis in obesity research that mesenteric fat is more metabolically active, and thus more likely to cause disease than subcutaneous depots [29••]. The implications of these two studies for the research of pharmacological intervention in obesity are apparent. Changes in adipose tissue dynamics, ie, reduced accumulation/increased breakdown, are the ultimate goal of any weight loss approach. New drugs targeting peripheral TG synthesis [31] rather than the central nervous system (or diet control) pathways further emphasize the need for biomarkers of adipose TG kinetics.

Strawford *et al* have further developed the ^2H_2O method to concurrently measure adipose TG synthesis, adipose cell proliferation and *de novo* lipogenesis in humans [32•].

Figure 13. Time course of label incorporation into the glycerol-moiety of adipose acyl-glycerides during ^2H_2O administration into different fat depots of adult rats.



Number of rats = 4. k_s Fractional synthesis rate, $t_{1/2}$ half-life, **d** days. *(Reproduced with permission from the American Physiological Society and Turner SM, Murphy EJ, Neese RA, Antelo F, Thomas T, Agarwal A, Go C, Hellerstein MK:* ***Measurement of TG synthesis and turnover in vivo by ^2H_2O incorporation into the glycerol moiety and application of MIDA.*** *Am J Physiol Endocrinol Metab (2003)* ***285****(4):E790-E803. © 2003 American Physiological Society.)*

Lipoprotein and cholesterol metabolism

Very low-density lipoprotein (VLDL) assembly, secretion and metabolism can readily be measured in rodents and humans using stable isotopes. In fact, key mechanistic advances in the field have come from kinetic studies. Although increased low-density lipoprotein (LDL) cholesterol clearance is the likely mechanism by which statins lower cholesterol, this is not currently used as a primary biomarker of drug efficacy. The reasons are 2-fold. Firstly, the broad acceptance of plasma cholesterol measurements, and their inexpensive and easy determination, understandably discourage the employment of new biomarkers. Secondly, the technical barriers to measuring LDL kinetics are not trivial when compared with plasma cholesterol measurements. Methods for determining plasma TG production in animals are similarly extremely labor intensive (intravenous infusions) or non-physiological (trition administration). Adaptation of the ^2H_2O labeling of adipose TG to plasma TG synthesis is promising in this regard [29••].

High-density lipoprotein (HDL) kinetics and reverse cholesterol transport (RCT) are complementary targets to VLDL/LDL. While techniques to determine HDL kinetics are available, they are similarly difficult to execute. The ultimate goal of modifying HDL kinetics is to increase RCT, which currently lacks a good kinetic metric. In light of the enormous public health, as well as economic importance of this area of medicine, new, robust and authentic biomarkers are needed.

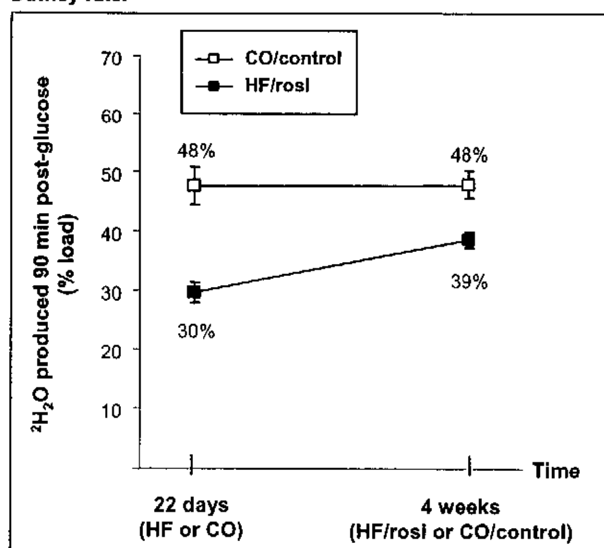
Insulin resistance and metabolic syndrome

Metabolic syndrome is increasingly recognized as a highly prevalent disease with profound economic and public health implications. Insulin resistance is widely believed to be the underlying etiology of metabolic syndrome and contributes to its natural history and pathology, as well as being a major risk factor for type 2 diabetes [33]. The potential health benefits to treating insulin resistance early are enormous, potentially reducing the risk of diabetes and cardiovascular disease. It is currently extremely difficult to measure insulin resistance. The gold standard approach is the glucose/insulin clamp, which is extremely labor intensive and impractical as a routine diagnostic tool or for use as a clinical or even preclinical tool to evaluate therapies. Other methods have emerged to replace the clamp. The HOMA and QUICKI tests are rapid and relatively easy to perform, but they suffer from large inter- and intra-individual variability, making them unsuitable for screening insulin-sensitizing agents. An alternative isotopic method has been reported by Lewanczuk *et al* using [^{13}C]glucose [34], involving the administration of trace amounts (25 mg) of ^{13}C glucose along with a small amount (15 g) of unlabeled glucose and then measuring the amount of expired $^{13}CO_2$ in the breath 90 min later. The authors propose that the recovery of breath $^{13}CO_2$ reflects glucose oxidation and therefore would correlate with impaired glucose disposal. While this assay provides improvements over HOMA or QUICKI (eg, no blood sampling and a single measurement), correlation with clamp results is relatively poor ($r^2 < 0.5$).

Murphy *et al* recently presented a new approach in which deuterated glucose was administered and the formation of deuterated water from the glycolytic metabolism of glucose was measured [35]. Both acquired (high-fat diet) and genetic

models (Zucker obese rats) of insulin resistance (IR) showed alterations using this test, and treatment with rosiglitazone reversed the abnormalities (Figure 14). Humans with established IR also exhibited alterations with this test. The availability of an accurate, high-throughput metric of IR, which can be used in preclinical as well as clinical DDD would represent an extremely useful tool for this important medical problem.

Figure 14. The effect of high-fat diet and rosiglitazone treatment on whole-body glycolytic disposal of [^{2}H]$_2$-glucose in Sprague-Dawley rats.



Rats were administered 2 mg/kg of 6,6-[^{2}H]$_2$-glucose by gavage and blood was collected from the tail vein after 90 min.
HF High-fat diet, **CO** control diet, **rosi** rosiglitazone treatment, **control** no rosiglitazone treatment.

Summary and conclusions

Kinetic biomarkers may offer the drug development field the ability to unclog the current bottleneck in the development of many promising compounds generated by the efficient modern lead discovery apparatus.

Technical improvements in MS over the last decade have enabled the use of many of the biomarkers discussed here. While beyond the scope of this review, major advances in MS continue to be developed for life-science applications. Non-dispersive infrared [36] and cavity ring-down isotope ratio spectroscopy [37], miniature cycloidal and GC-sector mass spectrometers are all being developed as sensitive, precise and affordable bench-top instruments. Not only might these new instruments improve on the cost and performance of existing bench-top mass spectrometers, they may also match detection limits of large, expensive isotope ratio mass spectrometers, opening up new and unexplored areas for kinetic tests.

Although there have been considerable advances, many areas of drug development still require kinetic biomarkers. In principle, a kinetic biomarker can be developed for any system in which the rate of synthesis or degradation of a protein, lipid, carbohydrate, ribonucleotide or cell is desired. Here we have surveyed many target pathways of interest to pharmaceutical research and noted the potential for applying stable isotope-based kinetic biomarkers. Table 1 comprises a partial list of high-priority kinetic biomarkers, which the authors believe are either particularly well-suited for kinetic solutions or are sufficiently difficult to study that few alternative methods are likely to be available.

In conclusion, new tools are required for *in vivo* drug development. The measurement of kinetics in intact systems offers researchers the ability to view the emergent regulatory processes of assembled and complex systems,

Table 1. High impact areas for kinetic biomarkers.

Field	Pathway	Rationale for a kinetic biomarker and therapeutic target
Cancer biology	Angiogenesis	Proliferation of endothelial cells is the hallmark of angiogenesis
	Pre-cancer chemoprevention	Reduction of the basal rate of cell proliferation may be an ideal target for chemoprevention
Metabolism	Islet/β-cell neogenesis	β-Cell death and regeneration are critical targets for type 1 and 2 diabetes treatments
Neurobiology	Remyelination	The synthesis and degradation of myelin are implicated in multiple sclerosis and other neurodegenerative diseases
	Amyloid precursor protein (APP)/Aβ-pathway	APP and Aβ-peptide synthesis are key targets in the prevention and delay of Alzheimer's disease
	Neuroinflammation	Inhibition of microglia proliferation may prevent inflammatory damage in the brain
	Cognition (learning/memory)	An assay of axonal plasticity (microtubule dynamics) may provide a biomarker of memory formation/loss
Inflammation/immunity	Fibrogenesis	Collagen synthesis/degradation is the ultimate target of antifibrotic treatments
	Joint space components	Abnormal turnover of glucosaminoglycans is implicated in the pathogenesis of arthritis
	Vaccination efficacy	The lifespan of memory T-cells is a measurable biomarker of vaccine efficacy
Toxicology	Liver cell death	A sensitive biomarker of liver cell death may detect potentially idiosyncratic liver toxins preclinically
Reproductive biology	Spermatogenesis	Spermatogenesis is a potential target for male infertility treatment
	Oogenesis	Stimulation of oogenesis is the 'holy grail' for female infertility treatment

and the effects of pharmacological interventions on pathways of interest. The rapid pace of instrument development and innovation make the potential range of applications of kinetics almost limitless. The challenge now is for the pharmaceutical industry to create *in vivo* biomarkers which address the critical pathways needed for the screening and evaluation of the drugs of the next century.

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