Expression of Insulin Receptor Isoform A and Insulin-like Growth Factor-1 Receptor in Human Acute Myelogenous Leukemia: Effect of the Dual-Receptor Inhibitor BMS-536924 In vitro

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Abstract
The insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF1R) are receptor tyrosine kinases that participate in mitogenic and antiapoptotic signaling in normal and neoplastic epithelia. In the present study, immunoblotting and reverse transcription-PCR demonstrated expression of IGF1R and IR isoform A in acute myelogenous leukemia (AML) cell lines as well as in >80% of clinical AML isolates. Treatment with insulin enhanced signaling through the Akt and MEK1/2 pathways as well as survival of serum-starved AML cell lines. Conversely, treatment with BMS-536924, a dual IGF1R/IR kinase inhibitor that is undergoing preclinical testing, inhibited constitutive receptor phosphorylation as well as downstream signaling through MEK1/2 and Akt. These changes inhibited proliferation and, in some AML cell lines, induced apoptosis at submicromolar concentrations. Likewise, BMS-536924 inhibited leukemic colony formation in CD34+ clinical AML samples in vitro. Collectively, these results not only indicate that expression of IGF1R and IR isoform A is common in AML but also show that interruption of signaling from these receptors inhibits proliferation in clinical AML isolates. Accordingly, further investigation of IGF1R/IR axis as a potential therapeutic target in AML appears warranted.

Introduction
The insulin-like growth factor (IGF) signaling pathway plays an important role in various cellular processes, including proliferation and differentiation (1–3). Components of this pathway include the ligands IGF1 and IGF2, which exhibit a high degree of sequence homology to insulin; IGF1 receptor (IGF1R), which contains an extracellular domain that binds both IGFs and an intracellular tyrosine kinase domain that initiates signaling; and IGF2 receptor, which lacks a tyrosine kinase domain and binds IGF2 without initiating signal transduction (1, 3, 4).

The IGF1R and insulin receptor (IR) pathways are closely related. IGF1R and IR exhibit extensive sequence similarity, including sequence identity in their ATP-binding clefs and at residues that contact the peptide ligands in their binding sites as well as 84% homology in the kinase domain (5). In both cases, ligand binding results in a conformational change that leads to autophosphorylation of the receptor tyrosine kinase domain; increased phosphorylation of the receptor substrates Shc, IRS-1 and IRS-2; and activation of the same downstream signaling cascades, including the mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt pathways (3–5). Indeed, IGF1R and IR are so similar that IGF1R and IR isoform A (IR-A), a splice variant that is overexpressed in certain carcinomas, can heterodimerize to form hybrid receptors that signal after binding IGF1, IGF2, or insulin at physiologic levels (6). Moreover, IR-A homodimers can bind IGF2 and, to a lesser extent, IGF1 (7–9), suggesting that proliferative IGF signaling can occur through IGF1R homodimers, IGF1R/IR-A heterodimers, and IR-A homodimers (6, 9). In contrast, hybrid receptors containing IGF1R and IR isoform B are orders of magnitude less sensitive to IGF2 and insulin (6).

Uncontrolled IGF1R signaling, which stimulates proliferation and protects cells from apoptosis (1, 3, 4, 10), has been implicated in the development and maintenance of various neoplasms (1, 3, 4, 11–13). As a result, the IGF1/IGF1R axis has become a target for anticancer drug development. Agents currently undergoing preclinical or clinical testing include neutralizing IGF1 antibodies, antagonistic IGF1R antibodies, and small-molecule IGF1R tyrosine kinase inhibitors (3, 4, 10, 14). In view of signaling by IGF1R/IR-A hybrids (6) and IR-A homodimers (7–9), as well as the high sequence homology of IGF1R and IR, there has also been recent interest in dual-receptor inhibitors. The dual IGF1R/IR inhibitor BMS-554417 exhibited antiproliferative and proapoptotic activity in vitro and in vivo with modest effects on glucose tolerance (15).

BMS-536924 (1H-benzoimidazol-2-yl-1H-pyridin-2-one), which is used in the present work, is likewise an ATP-competitive inhibitor of IGF1R and IR that has shown activity against neoplastically transformed cell lines (16, 17).

Although the role of the IGF1 system has been extensively investigated in various solid tumors, less is known about the role of IGF1R and IR in acute myelogenous leukemia (AML). Earlier reports showed that IGF1 enhances colony formation by committed normal myeloid and erythroid progenitors (18, 19), but more primitive CD34+ normal progenitors lack IGF1R expression and are resistant to IGF1R down-regulation (20). In addition, IGF1 enhances proliferation of human AML cell lines (21–24) and clonogenic growth of AML progenitors in vitro (25–27). More recently, expression of IR (28) as well as IGF1R (28, 29) was shown in AML cell lines and a handful of primary AML specimens. In

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addition, it was reported that insulin and IGF1 both enhanced the proliferation of AML cells and that down-regulation of either IR or IGFR modestly diminished proliferation of U937 cells (28), raising the possibility that IR signaling also contributes to survival and proliferation of AML cells. To build on these results, the present studies were designed to determine the frequency of IGFR1 and IR expression in a larger series of AML specimens, establish the identity of the IR splice form expressed in AML cell lines and clinical specimens, and assess the effect of the dual IGFR1/IR inhibitor BMS-536924 on AML cell lines and clinical AML specimens in vitro.

Materials and Methods

Materials. Antibodies that recognize the β-chains of IR (products 3020 and 3025) and IGFR1 (product 3027) as well as phospho-Tyr1146-IR (product 3021), phospho-Ser473-Akt, Akt, phospho-extracellular signal-regulated kinase (ERK) 1/2, and ERK1/2 were obtained from Cell Signaling Technology. Oligonucleotides were purchased from Integrated DNA Technologies. MTS and phenazine methosulfate were purchased from Promega and Sigma-Aldrich, respectively. All other reagents were obtained as described previously (30, 31).

Tissue culture. AML lines were propagated at densities of <1 × 10⁶ cells/ml in medium containing 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2% (v/v) fetal bovine serum. HL-60 cells (American Type Culture Collection), ML-1 cells (Michael Kastan, St. Jude Children’s Hospital), and K562 cells (Robert Abraham, Wyeth Pharmaceuticals) were maintained in medium A (RPMI 1640 with 10% (v/v) heat-inactivated fetal bovine serum). U937 cells (American Type Culture Collection) were maintained in medium A supplemented with 10 mmol/L HEPES (pH 7.4), 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose (medium B). All experiments with BMS-536924 were performed in these serum-containing medium.

MTS assay. Log-phase cells were sedimented at 100 × g for 5 min, washed once in serum-free media, and resuspended in serum-free medium. Aliquots containing ~5 × 10⁶ cells in 120 μl were incubated at 37°C with varying concentrations of insulin for 24 to 72 h. Alternatively, cells in medium A or medium B containing fetal bovine serum were incubated with varying concentrations of BMS-536924 for 5 days. After reaction with MTS and phenazine methosulfate as instructed by the suppliers, plates were incubated for 3 to 6 h to obtain an absorbance of >0.5 at 490 nm in control samples.

Detection of apoptosis. Flow cytometry for DNA content (31, 32) and fluorescence microscopy for apoptotic nuclear morphologic changes (33) were performed as described (31, 33, 34).

Clonogenic assays. Aliquots containing 500 to 1,000 HL-60 or U937 cells were plated in gridded 35-mm plates in the medium of Pike and Robinson (35) containing 0.3% (w/v) agar and the indicated concentrations of BMS-536924. After incubation for 10 to 14 days, colonies containing ≥50 cells were counted. Colony-forming assays in MCF-7 cells were performed as described (30).

Leukemia samples. Under the aegis of institutional review board-approved protocols, marrow samples were acquired from consenting patients with newly diagnosed AML before induction therapy. Mononuclear cells were isolated on Ficoll-Hyphaque gradients, washed with RPMI 1640, and resuspended at 1.5 × 10⁶/ml in Iscove’s modified Dulbecco’s medium containing 20% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2% (v/v) fetal bovine serum (medium C). Aliquots containing 600,000 cells were plated in gridded 35-mm culture dishes containing increasing BMS-536924 concentrations in growth factor-containing Methocult methylcellulose medium (StemCell Technologies), incubated for 14 days, and scored at ×25 magnification according to published morphologic criteria (36).

Reverse transcription-PCR. After total RNA was isolated using a RNeasy mini kit (Qiagen), cDNAs were synthesized using a SuperScript III First-Strand Synthesis kit (Invitrogen) following the supplier’s instructions. PCRs (50 μl) were completed using 3 μl cDNA product (5 μl for IR amplification), Promega Master Mix PCR reagents, and the primers listed in Supplementary Table S1. Amplification for IGFR1, IGFR2, IGFR1, IRS-2, and glyceraldehyde-3-phosphate dehydrogenase involved a three-step program: 95°C for 60 s; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and 72°C for 7 min. Amplification of IR and IRS-1 was accomplished by nested PCR using the outer primers according to the conditions described above and then reamplifying 2% of the first PCR using the inner primers listed in Supplementary Table S1. Alternatively, amplification of IR nucleotides 2,230 to 2,867, a region that contains exon 11 in isoform B but not isoform A, was completed using primers and conditions specified by Pandini and colleagues (6). Products were electrophoresed on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide in 1× TAE buffer (30.7 mmol/L Tris, 20 mmol/L sodium acetate, and 1 mmol/L EDTA), visualized on a UV transilluminator, excised, and sequenced using automated dye terminator technology.

Immunoprecipitation. All steps were performed at 4°C. Log-phase cells were washed twice in PBS and lysed by incubation for 20 min in buffer consisting of 150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.5), 10% (w/v) glycerol, 5 mmol/L MgSO₄, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 1% (w/v) thiodiglycol, and 1% (w/v) Triton X-100. After lysates were sedimented at 16,000 × g for 5 min, supernatants were incubated for 16 h with rabbit monoclonal anti-IR or polyclonal anti-IGFR1 on an end-over-end shaker. After addition of 50 μL of a 50% (v/v) suspension of protein A-Sepharose beads, samples were incubated for an additional 2 h. Receptor-antibody complexes were recovered by sedimentation at 4,000 × g for 1 min, washed four times with wash buffer [150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5), 10% (w/v) glycerol, 0.1% (w/v) Triton X-100, 1% (w/v) thiodiglycol, and 1 mmol/L sodium orthovanadate], eluted in SDS sample buffer, and subjected to SDS-PAGE followed by immunoblottting.

Immunoblotting. Whole-cell lysates were prepared from cell lines or clinical AML samples as described previously (37). Aliquots were resuspended in SDS sample buffer at 5 mg protein/ml (assayed by the bicinchoninic acid method), separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies as described (38).

Results

IGFR1, IR-A, and IGF1 are expressed in AML cell lines. To assess expression of IGFR1 and IR in human AML cells, reverse transcription-PCR and immunoblotting were performed using RNA and protein, respectively, from the HL-60, U937, and ML-1 human AML cell lines. As indicated in Fig. IA, reverse transcription-PCR readily detected message encoding IGFR1, IR, and the downstream signaling molecules IRS-1 and IRS-2 in each line. Sequencing demonstrated that the IR transcript in all three AML lines corresponds to isoform A (Fig. IA, right; and data not shown), an isoform that can bind IGF1 and IGF2 (7–9) as well as alter the ligand specificity of IGFR1 (6). Immunoblotting (Fig. IB) likewise demonstrated signals for IGFR1 and IR at ~100 kDa, the expected molecular weight for the mature β chain of each receptor, in all three lines. Importantly, IGFR1 was expressed at high levels in U937 and ML-1 cells, whereas IR was expressed at higher levels in HL-60. Immunoprecipitation followed by immunoblotting confirmed the association of a portion of the total IR with IGFR1 in HL-60 and U937 cells (Fig. IC, lanes 2 and 4), suggesting the presence of IGFR1/IR-A heterodimers as well as IR-A homodimers depending on the cell line. Analysis using affinity-purified antibodies that recognize a conserved autophosphorylation site on IGFR1 and IR also indicated that one or both receptors were phosphorylated under normal growth conditions (Fig. IB and C, top). As indicated in
Fig. 1D, message for IGF1 was detectable in HL-60 cells but not in the U937 or ML-1 cell lines. IGF2 mRNA was not detectable in any of the three AML lines.

To determine whether these receptors are functional, cells were treated with IGF1, IGF2, or insulin. Activating phosphorylations of ERK and Akt were readily observed within 5 min after addition of 10 nmol/L IGF1 or 13 nmol/L IGF2 (Fig. 2A), indicating that the IGF1R signals in response to physiologic ligand concentrations. Importantly, pathway activation was also observed within <1 min after insulin addition, suggesting that the IR expressed in these cells can signal (Fig. 2B). In further experiments, insulin at concentrations as low as 10 ng/mL (1.6 nmol/L) enhanced the reduction of MTS, a common assay of viable cell mass, after serum deprivation (Fig. 2C). The extent of this effect varied with the cell line, being highest in HL-60 cells (Fig. 2C). Because of concern that the MTS assay might reflect changes in glucose metabolism rather than cell viability, cell survival was also determined when cells were incubated with insulin, the IGF1 derivative LR-3, or IGF2 under serum-free conditions. As indicated in Fig. 2D for U937 cells, cell division continued over the first 24 h after serum withdrawal, reflecting completion of the cell cycle in the population that had passed the restriction point. Although cells subsequently died, LR-3 and particularly IGF2 increased survival relative to diluent-treated cells. This effect was evident on day 3 and, to a lesser extent, day 4.

Collectively, the results presented in Figs. 1 and 2 indicate that human AML cell lines synthesize IR-A as well as IGF1R, that IGF1R/IR-A heterodimers can be detected in some of these cells, and that these receptors can signal under normal growth conditions.

BMS-536924 inhibits receptor signaling and proliferation in AML cell lines. In light of the preceding results, we next assessed the effect of the dual IGF1R/IR inhibitor BMS-536924 (16) on receptor autophosphorylation and downstream signaling. As illustrated in Fig. 3A for ML-1 cells, submicromolar BMS-536924 concentrations induced a dose-dependent decrease in receptor phosphorylation of the two receptor kinases, indicating inhibition of receptor signaling.

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phosphorylation that was accompanied by inhibition of Akt and, to a lesser extent, ERK phosphorylation (Fig. 3A). Interestingly, IGF1R levels increased in response to BMS-536924, possibly reflecting a feedback pathway.

Subsequent experiments assessed the effect of BMS-536924 on AML cell proliferation and viability. When HL-60, U937, or ML-1 cells were incubated with BMS-536924 for 5 days, a dose-dependent decrease in viable cell mass was observed (B-D), with mean IC₅₀...
values ranging from 360 ± 130 nmol/L (mean ± SD, n = 3) for ML-1 cells to 1,800 ± 630 nmol/L for U937. Because this decrease can reflect either diminished proliferation or increased apoptosis, cells were also treated with BMS-536924 and subjected to flow cytometry. As illustrated in Fig. 4A for ML-1 cells, BMS-536924 induced readily apparent G1 arrest. In addition, cells with "subdiploid" DNA content accumulated (Fig. 4A-D), particularly in ML-1 cultures. Hoechst staining also showed the presence of cells with fragmented nuclei after exposure to BMS-536924 for 5 days (Fig. 4D, inset), confirming the induction of apoptosis.

To provide an alternative method for assessing the cumulative effects of BMS-536924, clonogenic assays were performed using HL-60 and U937 cells, the two lines that form colonies in soft agar. BMS-536924 diminished colony formation in both cell lines, with IC50 values of 340 ± 50 nmol/L (mean ± SD, n = 3) in HL-60 cells and <80 nmol/L (n = 3) in U937 cells (Fig. 5A and B). In comparison, MCF-7 breast cancer cells, which are considered an IGF1–dependent solid tumor line (17, 39–41), exhibited an IC50 of 500 ± 100 nmol/L (n = 3) in colony-forming assays (Fig. 5C), indicating that BMS-536924 induces long-term antiproliferative effects in HL-60 and U937 cells at concentrations similar to those that inhibit proliferation of IGF1–dependent solid tumor cell lines.

**Analysis of primary AML specimens.** In view of the effects of BMS-536924 on human AML cell lines, we next examined IGF1R, IR, and IGF expression in clinical AML specimens. In a group of 21 pretreatment AML specimens (Fig. 6A and data not shown), mRNA encoding IGF1R, IR, and IGF1 was detectable in 18 (86%) and mRNA for IGF2 was detectable in 15 (71%). When the IR message from the 18 positive AML samples was sequenced, only message encoding IR-A was detected (Fig. 6B and data not shown), as in the AML cell lines (Fig. 1A). In further experiments, blasts from 29 pretreatment AML marrows encompassing all FAB subtypes were examined by immunoblotting with antibodies that recognize IGF1R and IR. Twenty-six of the 29 (90%) samples expressed IGF1R and 25 of 29 (86%) expressed IR at the protein level (Fig. 6C). Interestingly, IGF1R and IR were readily detected by immunoblotting in samples that contained low but detectable message levels (Fig. 6A, right), suggesting a poor correlation between mRNA and protein for these two receptors.

Because the majority of the leukemic marrow samples expressed IGF1R and/or IR-A, we examined the effect of BMS-536924 on blast cell proliferation using clonogenic assays. For these studies, nine CD34+ AML samples were examined. As indicated in Fig. 6D, blast cells in these specimens exhibited a dose-dependent decrease in colony formation on exposure to BMS-536924, with IC50 values ranging from 1 to 7 μmol/L.

**Discussion**

Results of the present study provide the first evidence that human AML cells express exclusively isoform A, the proliferative form of the IR. Additional experiments show that the dual IGF1R/IR inhibitor BMS-536924 decreases autophosphorylation of its target receptors, diminishes signaling through the phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase pathways,
and inhibits proliferation of AML cell lines and colony formation in clinical AML samples. Collectively, these results identify the IGF1R/IR axis as a possible contributor to AML cell survival and show the potential utility of targeting both receptors in AML.

In earlier work, Tazzari and colleagues reported that IGF1 expression and IGF1R phosphorylation were increased in blast cells at the time of relapse in four AML patients (29). Doepfner and colleagues subsequently showed expression of IR in addition to IGF1R in AML blasts (28). The present study extended these observations by showing that both IR and IGF1R are expressed at protein and RNA levels in >80% of AML samples (Fig. 6A). Although the present study was not designed to rule out a possible contribution to these signals from small numbers of contaminating stromal cells, the presence of IGF1R and/or IGF2 message in a variety of AML lines, including HL-60, K562, THP-1, KG1a, and HEL (Fig. 1D; data not shown), indicates that myeloid cells can express these ligands. Coupled with recent reports that these ligands are secreted by clinical AML samples (28, 42), these results raise the possibility of an autocrine or paracrine pathway involving IGF1R and IR-A in AML. In view of the ability of IGF1R and IR-A ligands to activate downstream signaling (Fig. 2A and B), such a pathway might contribute to the previously described constitutive activation of the mitogen-activated protein kinase (43–45) and phosphoinositide 3-kinase/Akt pathways (46–48) in clinical AML specimens.

In further experiments, BMS-536924, an example of a new drug class that inhibits IGF1R and IR almost equally (15, 16), diminished proliferation at concentrations as low as 200 nmol/L (Fig. 3B–D) and induced apoptosis in susceptible AML lines (Fig. 4). Moreover, BMS-536924 inhibited colony formation in CD34+ clinical AML samples by as much as 98% (Fig. 6D). There was, however, variability among clinical samples as indicated by the fact that 10 µmol/L BMS-536924 inhibited colony formation by 60% to 98% (Fig. 6D).

This variability requires further investigation. No clear-cut correlation between different AML subtypes and BMS-536924 sensitivity is evident from the present studies, although the sample size was extremely small. On the other hand, we have observed differences in expression of some of the proteins that might be predicted to affect sensitivity, including IGF1R, IR, IRS-1, and IRS-2 (Fig. 6C; data not shown). Because expression of these polypeptides in the total blast population might not reflect levels in the cells that form colonies, it will be important to examine these polypeptides in specific AML subpopulations in the future. Other factors that might
Contribute to sensitivity differences include variability in expression of antiapoptotic Bcl-2 family members (15) or the transporter ABCG2, which has recently been shown to efflux BMS-536924 (49). Finally, it is conceivable that the ability or inability of cells to up-regulate IGF1R and/or IR upon pathway inhibition might contribute to the variability. Future experiments will need to determine whether the BMS-536924-induced IGF1R up-regulation observed in Fig. 3A reflects increased gene expression, message stabilization,

Figure 5. BMS-536924 inhibits colony formation in AML cell lines. Log-phase HL-60 (A), U937 (B), or MCF-7 (C) cells were plated in medium containing 0.3% agar and varying concentrations of BMS-536924 (A and B) or allowed to adhere to tissue culture plates and continuously exposed to BMS-536924 (C). After a 10- to 14-d incubation, colonies were counted. Points, mean of quadruplicate samples; bars, SD.

Figure 6. Examination of the IGF1R/IR axis and effects of BMS-536924 on colony formation in clinical AML samples. A, blasts from untreated AML patients were subjected to reverse transcription-PCR with primers for the indicated message. Glyceraldehyde-3-phosphate dehydrogenase served as a positive control. Lanes 1 to 9, nine separate AML patients. Inset, samples from patients 3 and 4 were subjected to 45 cycles of amplification (top) or immunoblotting for the indicated antigen (bottom). B, chromatograms obtained when IR cDNAs from patients 2 and 5 were sequenced across the splice junction between exons 12 and 11 (dashed line). Shown above the chromatogram are the predicted sequences of isoforms A and B. Arrows, nucleotides unique to A isoform. C, whole-cell lysates from an additional patient cohort were subjected to immunoblotting with antibodies that recognize the indicated antigen or, as a control, histone H1. D, AML samples used in A were plated in Methocult containing the indicated BMS-536924 concentration. Leukemic colonies were quantitated after 14 d as instructed by the supplier. Each line represents one clinical sample. Colors of lines indicate corresponding sample in A. Sample 7 in A did not form colonies. Conversely, the sample shown in black in D did not have enough cells for RNA isolation. Open symbols, sample with activating FLT3 mutation. Absence of lines beyond 10 μmol/L BMS-536924 indicates lack of colonies at higher concentrations in several samples.
and/or post-translational changes, whether similar effects are observed in clinical leukemia specimens, and whether this up-regulation affects drug sensitivity.

Additional studies are also required to determine whether a dual IGF1R/IR inhibitor such as BMS-536924 exhibits selectivity for AML compared with normal progenitors. It is known that primitive CD34+ normal progenitors lack IGF1R expression and are resistant to IGF1R down-regulation (20). Although it has been suggested that IR signaling might provide a survival signal for normal CD34+ cells (50), the paucity of marrow toxicity in treated with the IGF1R/ IR inhibitor BMS-554417 (15), coupled with the limited effects of NVP-AEW541 on normal human CD34+ progenitors (28, 29) at concentrations that inhibit IR in addition to IGF1R, suggests that effects of dual IGF1R/IR inhibition on normal myeloid progenitors will be modest.

In summary, the present results not only show that IR-A, like IGF1R, is widely expressed in AML cell lines and clinical samples, but also show that the dual IGF1R/IR inhibitor BMS-536924 induces apoptosis in AML cell lines and profoundly inhibits colony formation in a subset of AML samples in vitro. Based on these results, further preclinical and possible clinical testing of therapies that simultaneously target IGF1R and IR in AML appear warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Insulin Receptor A and IGF1R in AML


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