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Synthesis and evaluation of a novel ^{99m}Tc-labeled bioreductive probe for tumor hypoxia imaging

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ABSTRACT

Tumor hypoxia is closely associated with the malignant progression and/or the high metastatic ability of tumors and often induces resistance to chemo- and/or radiotherapy. Thus, the detection and evaluation of hypoxia is important for the optimization of cancer therapy. We designed a novel ^{99m}Tc-labeled probe for tumor hypoxia imaging that utilizes bioreductive reactions in hypoxic cells. This probe, which contains a 4-nitrobenzyl ester group, is reduced in hypoxic cells to produce a corresponding carboxylate anion that cannot penetrate cell membranes because of its hydrophilicity and negative charge; therefore, it is expected to be trapped inside hypoxic cells. Based on this unique strategy, we synthesized the Technetium-99m (^{99m}Tc)-labeled probe ^{99m}Tc-SD32. The uptake of ^{99m}Tc-SD32 in tumor cells was investigated under normoxic and hypoxic conditions. ^{99m}Tc-SD32 showed sufficient accumulation and good retention in hypoxic cells. In addition, we demonstrated that ^{99m}Tc-SD32 was subjected to bioreduction in hypoxic cells and was trapped as the corresponding carboxylate anion. These results indicated that ^{99m}Tc-SD32 would be a promising agent for in vivo hypoxia imaging.

Hypoxic areas induced by incomplete vascular networks[1] and resultant alteration between the supply and consumption of oxygen[2] are commonly observed in solid tumors regardless of the type of cancer. These sites play a pivotal role in the malignant progression and/or high metastatic ability of tumors.[3–5] Tumor cells located in such regions are often resistant to chemo- and/or radiotherapy and diminish the beneficial effects of cancer therapy.[6] Therefore, non-invasive visualization and quantitative evaluation of tumor hypoxia will contribute greatly to the optimization of cancer therapy. To date, various hypoxia imaging radiopharmaceuticals[7] have been developed and some have been used in clinical settings. Most are tracers for positron emission tomography (PET) such as [^{18}F]FMISO,[8,9] [^{18}F]FAZA,[10] and [$^{62/64}$Cu]Cu-ATSM.[11–13] However, the use of PET tracers bearing cyclotron radionuclides is limited because of their extremely short half-life and high cost. On the other hand, Technetium-99m (^{99m}Tc), a pure gamma-emitting radionuclide with optimal nuclear properties for single photon emission computed tomography (SPECT), is easily obtained with a ^{99}Mo/^{99m}Tc generator system and have been used for various types of diagnostic tracers in clinical situations.[14] Herein, we report the design, synthesis and in vitro evaluation of a novel ^{99m}Tc-labeled probe bearing simple strategy of metabolic trapping in hypoxic cells for tumor hypoxia imaging.

Our strategy to retain ^{99m}Tc-labeled probes in hypoxic cells is shown in Figure 1. This probe **1** comprises a 4-nitrobenzyl ester moiety and mercaptoacetyltriglycine (MAG3) known as N3S1 chelator. Nitro aromatic groups such as nitroimidazoles[15] or nitrobenzenes[16] are typical substrates for reducing enzymes in hypoxic cells. 4-Nitrobenzyl ester is also expected to be reduced in hypoxic cells. Following reduction, this ester forms the corresponding hydroxylamine (or amine) and is subsequently eliminated because of its electron donating effect: consequently, it is converted to a carboxylate anion.[17] The sequential reduction and elimination of 4-nitrobenzyl group occurring in hypoxic cells should induce the considerable changes in hydrophilicity. In other words, 4-nitrobenzyl ester **1** is relatively lipophilic and permeable to cell membranes, whereas the corresponding carboxylic anion **2** generated by bioreduction in hypoxic cells is significantly hydrophilic and not permeable to cell membranes under physiological conditions. Thus, it is retained within hypoxic cells. Various hypoxia imaging agents derived from nitroimidazole[8–10,18–22] have been developed. Their retention mechanisms are believed to be based on their ability to bind cytoplasmic proteins and/or DNAs following reduction,[15] but the exact mechanism has not yet been elucidated. We synthesized and evaluated a novel ^{99m}Tc-labeled hypoxia probe, which is distinctively different from those nitroimidazole derivatives.

A ligand bearing a 4-nitrobenzyl ester moiety, SD32, was constructed using Boc chemistry as shown in Scheme 1. The reaction of Boc-glycine **3** with 4-nitrobenzyl alcohol **4** in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) produced the corresponding ester **5**. The Boc group of **5** was removed by treatment with 95% trifluoroacetic acid (TFA), followed by acylation of the resulting

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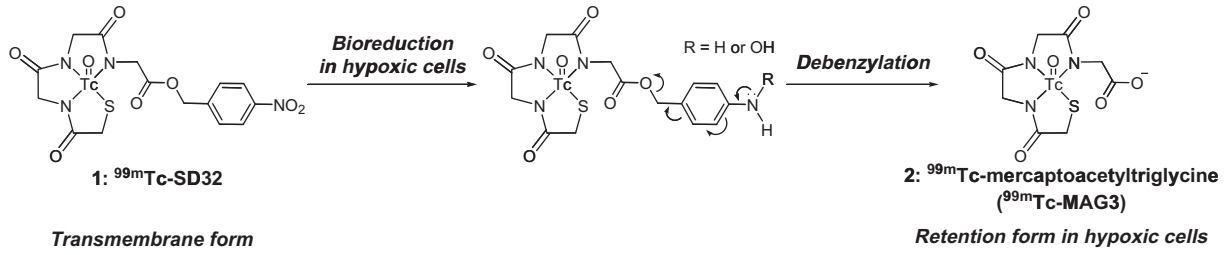
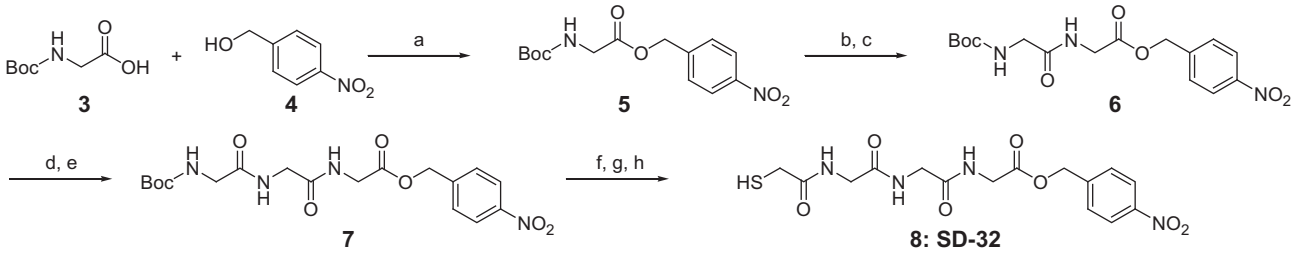


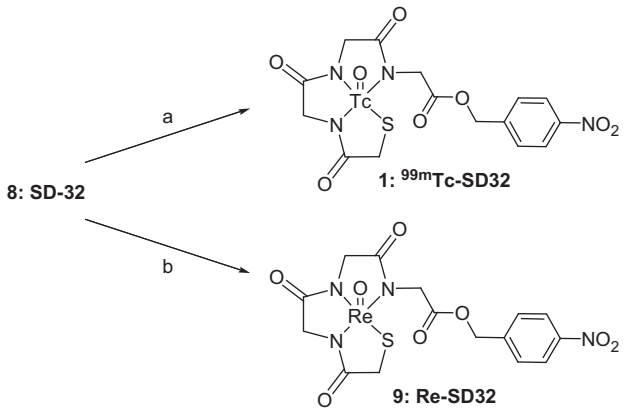
Figure 1. Molecular design of a ^{99m}Tc probe for hypoxia imaging.



Scheme 1. Reagents and conditions: (a) EDCI·HCl, DMAP, DCM, TEA, rt, 96%; (b) 95% TFA; (c) **3**, EDCI·HCl, HOBt, TEA, DMF, 96%; (d) 95% TFA; (e) **3**, EDCI·HCl, HOBt, TEA, DMF, 77%; (f) 95% TFA; (g) TrtSAcOH, EDCI·HCl, HOBt, TEA, DMF; (h) TFA, H_2O, EDT, TIPS, 48% (over 3 steps).

amino group using in situ activation of the carboxylic acid of **3** with EDCI, 1-hydroxybenzotriazole (HOBt) and triethylamine (TEA) to afford **6**. The second glycine was coupled in the same manner as **6** to afford **7**. Deprotection of **7**, acylation with *S*-trityl mercaptoacetic acid (TrtSAcOH), and the final trityl deprotection produced the desired ligand, **8**: SD32.

The complex formation of ^{99m}Tc- and Re-SD32 is summarized in Scheme 2. SD32 was labeled with ^{99m}Tc by a ligand-exchanging reaction using ^{99m}Tc-glucoheptonate that was synthesized in advance from the reaction of Na[$^{99m}TcO_4$] with Sn(II)-glucoheptonate complex. Purification by RP-HPLC on a C18 column eluted with acetonitrile/10 mM ammonium acetate (pH 7) afforded **1**: ^{99m}Tc-SD32 with a 76% radiochemical yield and >99% radiochemical purity. For analyzing the structure of ^{99m}Tc-SD32, the corresponding rhenium complex, **9**: Re-SD32 was synthesized by the reaction of SD32 with tetrabutylammonium tetrachlorooxorhenate (V) in DMF containing 5% methanol and 1% pyridine (Scheme 2).[21] Proton and carbon-13 NMR spectral data of SD32 and Re-SD32 were analyzed by H–H COSY, HMQC, and HMBC to determine the structure of Re-SD32 (Tables S1 and S2). The three amide carbonyls, C(4), C(7), and C(10) on Re-SD32 were considerably shifted to a lower field compared to those on SD32; however, ester carbonyl C(1) signal did not show a significant chemical shift change. Moreover, two methylene protons at the H(5) position of Re-SD32 were separated by fixing the conformation of the ligand moiety, though those of SD32 were symmetric and duplicated. The other methylene protons at the H(2), H(8), and H(11) also showed the same separation patterns. Additionally, high resolution mass spectrometry in the negative mode confirmed that the molecular formula of Re-SD32 was $C_{15}H_{14}N_4O_8SRe$. These NMR and HR-MS results showed that oxorhenium (V) was coordinated on a sulfur atom and three amide nitrogens as shown in Scheme 2. X-ray crystallographic analysis of Re-[23] and Tc-MAG3[24] were independently reported, and these structures were identical. The chelating moiety of SD32 was thought to be identical to MAG3; therefore it appears that the structures of the two metal complexes Tc- and Re-SD32 are also identical. In fact, RP-HPLC analysis showed the peaks for ^{99m}Tc-SD32 and Re-SD32 were observed at 10.0 and 9.7 min, respectively, (Fig. S1) and the similar retention times of these complexes supports their similarity.[21]



Scheme 2. Reagents and conditions: (a) ^{99m}Tc-glucoheptonate, 50 °C, 76%; (b) TBA[$ReOCl_4$], methanol, pyridine, DMF, 85 °C, 65%.

Cellular uptake and retention of ^{99m}Tc-SD32 in hypoxic and normoxic cells was studied using the FM3A murine breast tumor cell line.[25] Cells were seeded into each well of 12-well culture plates and incubated in advance at 37 °C under normoxic (95% air, 5% CO_2) or hypoxic (95% N_2, 5% CO_2) conditions for 1 h,[26] then ^{99m}Tc-SD32 (0.3 MBq/well) was added and the plates incubated under their respective culture conditions. At various time points (5, 15, 30, 60, and 90 min) following the addition of ^{99m}Tc-SD32, cell suspensions were filtered by vacuum filtration using membrane filter to collect cells, and their radioactivity was measured. All experiments were performed in triplicate. Figure 2 shows time-dependent change of cellular uptake and retention of ^{99m}Tc- labeled probes in hypoxic and normoxic cells. It was revealed that ^{99m}Tc radioactivity was retained specifically in hypoxic cells. That is, ^{99m}Tc radioactivity in the hypoxic cells increased time-dependently up to 60 min and then reached the plateau. The peak accumulation value (26.0–26.3%) was approximately 2.4 times higher than the initial uptake (10.9%). On the other hand, the uptake of ^{99m}Tc-SD32 in normoxic cells did not increase time-dependently although its initial uptake was similar to that in hypoxic cells. As for the low uptake in normoxic cells, we examined whether it could be washed out from the cells

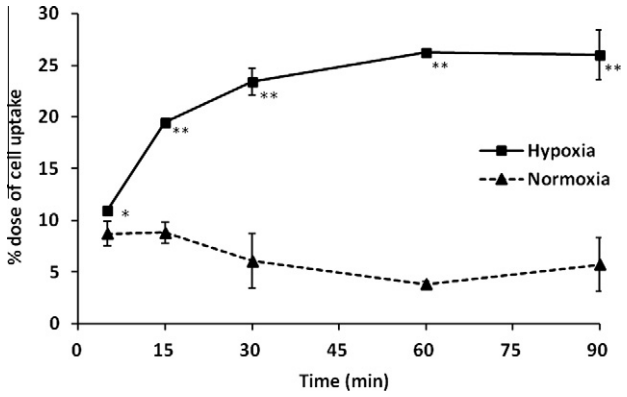


Figure 2. Cellular uptake and retention of ^{99m}Tc-SD32 under normoxic and hypoxic conditions. Values are mean ± standard deviation. $^{*}p$ <0.05, $^{**}p$ <0.01 versus normoxia by Tukey's test.

(Fig. 3). After incubating hypoxic and normoxic cells with ^{99m}Tc-SD32 for 1 h as described above, the medium was replaced with ^{99m}Tc-SD32-free medium, and these cells were incubated for an additional 3 h under both hypoxic and normoxic conditions. Under hypoxic condition, no significant wash out was observed following additional incubation. On the other hand, the radioactivity in normoxic cells after additional 3-h-incubation was decreased to 60% compared with the value just after the replacement of medium. These results strongly suggest that ^{99m}Tc-SD32 was trapped in hypoxic cells because of its conversion to hydrophilic compounds following reduction in hypoxic cells, while ^{99m}Tc-SD32 was washed out from normoxic cells with no degradation. In fact, after 1-h-incubation with ^{99m}Tc-SD32 in both hypoxic and normoxic conditions, ^{99m}Tc-radiocompound found in these culture media was only ^{99m}Tc-SD32 itself and no degradation products were observed by HPLC analyses (data not shown).

To characterize the structure of ^{99m}Tc complexes retained in hypoxic cells, we analyzed the cell lysates by HPLC (Fig. 4). After the incubation with ^{99m}Tc-SD32 under hypoxic or normoxic conditions, these cells were collected and lysed. The lysates were applied to ODS column and eluted with 10 mM ammonium acetate/MeCN. ^{99m}Tc-SD32 as a standard was also eluted under the same condition. The peak for ^{99m}Tc-SD32 was found at 10.0 min (Fig. 4a). In the analysis of hypoxic cell lysate, the ^{99m}Tc-SD32 peak almost disappeared, and instead, a new peak appeared at 2.0 min (Fig. 4b). This considerable shift in retention time indicated a significant change in hydrophilicity, that is, lipophilic ^{99m}Tc-SD32 was converted to a hydrophilic compound. On the other hand, no significant change in normoxic cell lysate was observed (Fig. 4c). In order to identify the hydrophilic peak at 2.0 min, ^{99m}Tc-SD32 was reduced in vitro in the presence of NADPH by nitroreductase from *Escherichia coli*, which is an oxygen-insensitive reducing enzyme of the nitro group,[16] and similarly analyzed. The retention time of the metabolites by the nitroreductase was observed at 2.0 min that was consistent with that of the hypoxic cell lysates (Fig. 4d). Moreover, ^{99m}Tc-MAG3, that is the debenzylated form of ^{99m}Tc-SD32, was found to exhibit the same retention time as that of hypoxic cell lysate and the reduced metabolite (Fig. 4e). These results strongly suggested that nitro group on ^{99m}Tc-SD32 was reduced and debenzylation occurred in hypoxic cells, and as a result, it was converted to ^{99m}Tc-MAG3 and accumulated in hypoxic cells. In addition, it was confirmed that almost all radioactivities applied on the column were recovered from the elution.

To analyze the reduction–debenzylation process of this probe in detail, the reduction of Re-SD32 by nitroreductase was monitored by LC/MS. Technetium does not have non-radioactive isotope; therefore, rhenium is often used as a non-radioactive analog of technetium to perform cold chemistry in various applications.[27] The reaction mixture at each time point (0.1, 5, 15, and 120 min) was analyzed by HPLC and their UV and MS chromatograms were obtained. Figure 5 shows the HPLC-UV chromatogram. The peak of Re-SD32 at 9.6 min decreased time-dependently and disappeared up to 120 min. Instead, a new peak at 1.4 min was appeared and gradually increased. This peak shift in retention time was consistent with that of ^{99m}Tc-SD32 as shown in Figure 4. HPLC–MS analyses

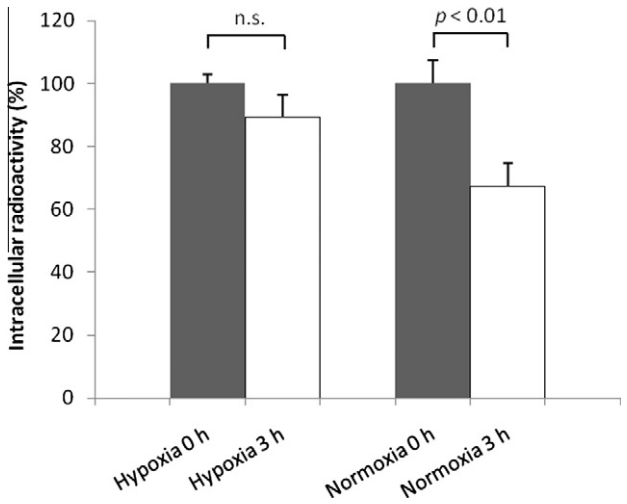


Figure 3. Intracellular radioactivity after substitution with ^{99m}Tc-SD32 free medium.

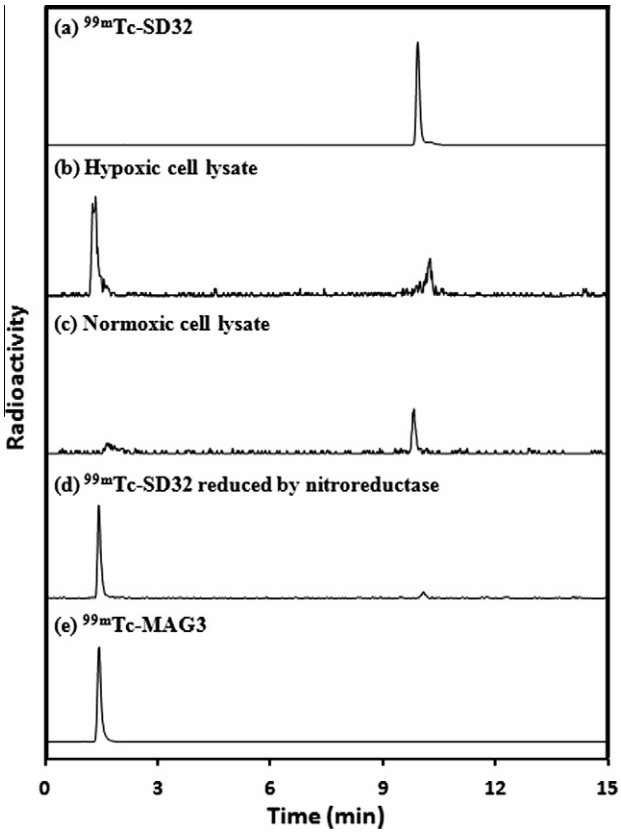


Figure 4. HPLC analysis: (a) ^{99m}Tc-SD32; (b) cell lysate from hypoxic cells incubated with ^{99m}Tc-SD32 for 1 h; (c) cell lysate from normoxic cells incubated with ^{99m}Tc-SD32 for 1 h; (d) ^{99m}Tc-SD32 reduced by nitroreductase; (e) ^{99m}Tc-MAG3.

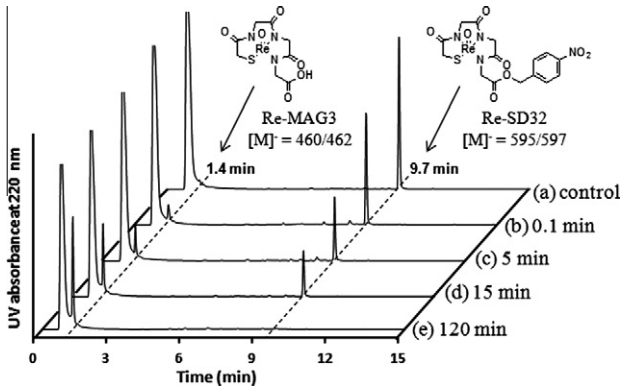


Figure 5. LC/MS analysis (a) Re-SD32 incubated with NADPH for 2 h (control); (b–e) Re-SD32 incubated with nitroreductase and NADPH for 0.1, 5, 15, and 120 min, respectively.

(shown in Fig. S2) provided further information on the structure of the metabolites. The ion peak corresponding to new UV peak at 1.4 min in Figure 5 showed negative ions of high intensity at m/z = 460/462 that is identical to the value of the debenzylated metabolite, Re-MAG3. Furthermore, two other ion peaks with high intensity were found in the mass chromatogram. These peaks were not detected in UV chromatogram. One peak at 9.8 min showed m/z = 579/581, and the other at 8.0 min showed m/z = 581/583. These molecular weights are consistent with that of nitroso and hydroxylamine derivatives of Re-SD32, respectively, and these products were presumed to be the reduction intermediates of Re-SD32. The existence of these intermediates as well as Re-MAG3 indicated that the conversion of Re-SD32 to Re-MAG3 proceeded via these intermediates. Thus, it was suggested that Re- and ^{99m}Tc-SD32 were subject to stepwise reduction to produce the corresponding carboxylic acids as shown in previous report[16] and they were not formed by simple hydrolysis.

The lipophilicity of ^{99m}Tc-SD32 was compared to that of reduced metabolite of ^{99m}Tc-SD32 and ^{99m}Tc-MAG3 by estimating their *n*-octanol/PBS (pH 7.4) partition coefficients. The values of partition coefficient for ^{99m}Tc-SD32, ^{99m}Tc-MAG3, and the reduction metabolite of ^{99m}Tc-SD32 were estimated by shake flask method, and their log*P* values at pH 7.4 were found to be 1.13, <−2, and <−2, respectively. In addition, the electrochemical properties of the three ^{99m}Tc-labeled compounds were evaluated by electrophoresis (Fig. S3). All compounds possessed negative charge, and the migration distance of ^{99m}Tc-MAG3 and the reduction metabolite of ^{99m}Tc-SD32 were identical. The migration distance of ^{99m}Tc-MAG3 was much longer than that of ^{99m}Tc-SD32 mainly because of di-anionic character of ^{99m}Tc-MAG3. These results on physiochemical properties showed that ^{99m}Tc-SD32 was relatively lipophilic compound in spite of mono-anionic property, while the reduced metabolite of ^{99m}Tc-SD32 and ^{99m}Tc-MAG3 were di-anionic and extremely hydrophilic. These drastic changes of physiochemical property would be contributed to the accumulation of ^{99m}Tc-SD32 in hypoxic cells.

In conclusion, we successfully designed and synthesized a novel ^{99m}Tc-labeled probe, ^{99m}Tc-SD32, for visualizing tumor hypoxia; this probe was specifically accumulated in hypoxic cells. ^{99m}Tc-SD32 was selectively reduced and retained in hypoxic cells, whereas it was washed out from normoxic cells without degradation. HPLC and LC/MS results demonstrated that the conversion of ^{99m}Tc-SD32 to the corresponding carboxylate anion was triggered by the bioreduction of the nitro group on ^{99m}Tc-SD32 in hypoxic cells as mentioned in its molecular design and not because of simple hydrolysis. The selective accumulation of ^{99m}Tc radioactivity in hypoxic cells is attributed to the physiochemical properties of the reduced metabolite of ^{99m}Tc-SD32. These results are encouraging for further in vivo SPECT imaging and continued exploration of ^{99m}Tc-labeled probes based on this molecular design.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.022.

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