Measurement of Tumor VEGF-A Levels with
89Zr-Bevacizumab PET as an Early Biomarker for the
Antiangiogenic Effect of Everolimus Treatment in an Ovarian
Cancer Xenograft Model

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Abstract

Purpose: The mTOR pathway is frequently activated in ovarian cancers. mTOR inhibitors, such as everolimus, can reduce VEGF-A production by cancer cells. We investigated whether early everolimus treatment effects could be monitored by positron emission tomography (PET) with 89Zr-bevacizumab.

Experimental Design: The effect of everolimus on VEGF-A secretion was determined in a panel of human ovarian cancer cell lines and in A2780skov3 ovarian cancer cells xenografted subcutaneously in BALB/c mice. Mice received daily 10 mg/kg everolimus intraperitoneally (i.p.) for 14 days. PET scans with the tracer 89Zr-labeled bevacizumab were conducted before and after treatment. Ex vivo 89Zr-bevacizumab biodistribution and correlative tissue analyses were conducted. Tumor VEGF-A levels were measured with ELISA and mean vascular density (MVD) was determined with immunohistochemistry.

Results: Everolimus treatment reduced VEGF-A levels in the supernatant of all cell lines. Everolimus lowered 89Zr-bevacizumab tumor uptake by 21.7% [mean standardized uptake value (SUV mean) 2.3 ± 0.2 vs. 2.9 ± 0.2, P < 0.01]. Ex vivo biodistribution also showed lower tracer uptake in the tumors of treated as compared with control animals (7.8 ± 0.8%ID/g vs. 14.0 ± 1.7%ID/g, P < 0.01), whereas no differences were observed for other tissues. This coincided with lower VEGF-A protein levels in tumor lysates in treated versus untreated tumors (P = 0.04) and reduced MVD (P < 0.01).

Conclusion: Tumor VEGF-A levels are decreased by everolimus. 89Zr-bevacizumab PET could be used to monitor tumor VEGF-A levels as an early biomarker of the antiangiogenic effect of mTOR inhibitor therapy.

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Introduction

Patients with ovarian cancer often present with advanced stage disease and develop resistance to conventional (platinum-based) chemotherapy during the course of treatment, resulting in a poor 30% 5-year survival rate (1). To improve ovarian cancer prognosis, there is a clear need for additional therapeutic options. Because ovarian cancers are often extensively vascularized and overexpress proangiogenic factors, such as VEGF-A, angiogenesis inhibition has been studied as a therapeutic strategy. Phase II and III trials with the VEGF-A–neutralizing antibody bevacizumab or with VEGF receptor (VEGFR)–targeted tyrosine kinase inhibitors (TKI) targeting vascular endothelial cells have shown anti-tumor activity in a subgroup of patients (2, 3).

The kinase mTOR is a potential alternative target for antiangiogenic therapy. The mTOR pathway is activated in 64% to 85% of ovarian cancers (4, 5). mTOR enhances translational efficacy of mRNAs that contain intricate 5′-untranslated regions (5′-UTR). These regions encode onco- genic proteins, such as hypoxia-inducible factor (HIF) and VEGF-A (6, 7). By reducing the synthesis of these proteins by tumor cells, mTOR inhibitors have distinct antiangiogenic effects as compared with classical antiangiogenic drugs, such as bevacizumab (8).

In transgenic (orthotopic) and xenograft mouse models, treatment with the mTOR inhibitor everolimus delayed tumor development, reduced tumor burden, and prolonged...
Translational Relevance

The mTOR is frequently activated in ovarian cancers and is involved in tumor angiogenesis. Drugs inhibiting mTOR are of interest as they can exert antitumor activity, which is in part executed by reducing VEGF-A production. Clinical studies indicate benefit of mTOR inhibition in a subgroup of patients with ovarian cancer, but biomarkers for patient selection are currently lacking. Our data show that $^{89}$Zr-bevacizumab positron emission tomography (PET) provides a novel tool for noninvasive monitoring of antiangiogenic effects upon mTOR inhibitor therapy. These results support the evaluation of measuring tumor VEGF-A levels with $^{89}$Zr-bevacizumab PET as an early biomarker for the antiangiogenic effect of mTOR inhibitors in clinical studies.

Materials and Methods

Cell lines

The human ovarian cancer cell line A2780 was kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The luciferase-transfected subline A2780luc was developed as described earlier (18). SKOV-3 and TOV-21G ovarian cancer cell lines were obtained from the American Type Culture Collection. A2780 and A2780luc cells were cultured in RPMI-1640, suplemented with 10% fetal calf serum (FCS, Bodinco BV) and 2 mmol/L l-glutamine (Invitrogen), SKOV-3 in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/L glucose, supplemented with 10% FCS, and TOV-21G cells in Ham-F12 and DMEM (1:1), supplemented with 10% FCS. All culture media were purchased from Invitrogen. All cell lines were cultured at 37°C in a fully humidified atmosphere containing 5% CO$_2$.

Compounds

Everolimus was obtained from LC Laboratories (E-4040, stored at −20°C). For in vitro use, everolimus was dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L stock concentration stored at −20°C. For in vivo use, everolimus was dissolved in DMSO and formulated in a stable suspension with PBS containing 0.5% Tween-80 (Sigma-Aldrich). Mice received 10 mg/kg everolimus daily via intraperitoneal injection (10 mg/kg). The everolimus dosage used is based on previous efficacy studies in xenografted mice, in which 5 to 10 mg/kg daily resulted in unbound (active) everolimus plasma levels comparable with those reached in humans receiving 5 to 10 mg daily due to higher plasma protein binding and shorter $t_{1/2}$ values in mice (8, 19–22).

Western blotting

Total and phosphorylated protein expression of mTOR target proteins was measured by Western blotting. After 24 hours of everolimus exposure, adherent cell layers were washed 3 times with ice-cold PBS, and total cellular protein content was extracted with mammalian protein extraction reagent (M-Per, Thermo Scientific). Protease and phosphatase inhibitors (Thermo Scientific) were added (1:100). Protein yield was determined with the Bradford assay (23). Samples were diluted 1:1 with SDS sample buffer, containing 125 mmol/L Tris–HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 1% β-mercaptoethanol, and boiled for 5 to 10 minutes before storage at −20°C. Proteins (20–30 μg) were separated on SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) by wet blotting (24). Primary antibodies were purchased from Cell Signaling, recognizing epitopes on p70S6K (pS705/pS65), phosho-p70S6K (Thr389; #9206), S6 (S235/236; #2217), phospho-S6 (Ser235/236; #4856), 4E-BP1 (#9644), and phospho-4E-BP1 (Thr70; #9455). Anti–β-actin antibody (BP Biomedicals) served to assure equal protein loading. Membranes were incubated with horseradish peroxidase (HRP)–labeled secondary antibodies (DAKO) for 1 hour at room temperature. Protein bands were visualized with chemiluminescence using Lumi-Light (Roche Diagnostics).
VEGF-A ELISA

After 24 hours incubation with everolimus, cell culture supernatant was removed and centrifuged for 10 to 15 minutes at 450 x g, to remove any residual cells or cell remnants, and subsequently stored in aliquots at −20°C until VEGF-A levels were measured with VEGF Quantikine ELISA kits (DVE00, R&D Systems). To correct for experimental differences, cell counting was conducted in parallel for each sample using conventional counting grids and Trypan blue staining.

For in vivo studies, 3 random cores were die-cut from frozen tumor samples and lysed using M-PER (16). In these whole-tumor protein lysates, VEGF-A levels were assayed with ELISA kits and total protein content with Bradford assays as described earlier.

Xenograft model

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, The Netherlands). Animal studies were conducted in male nude BALB/c mice (BALB/cOlaHsd-Foxn1nu, Harlan). Animals were allowed to feed ad libitum. Experiments were carried out under isoflurane inhalation anesthesia (induction 3%, maintenance 1.5%).

Mice (n = 10) were xenografted subcutaneously in the flank with 5 × 10⁶ A2780huC cells resuspended in 0.3 mL of a 1:1 mixture of PBS and Matrigel (BD Biosciences). To ensure tumor viability, bioluminescence imaging was conducted using the IVIS Spectrum system (Caliper Life Sciences). D-luciferin (150 mg/kg, purchased from Xenogen) was reconstituted in PBS and injected intraperitoneally 20 minutes before image acquisition. Twelve days after tumor inoculation, when the tumor diameter measured 6 to 8 mm (~300 mm³), a baseline 6-day microPET scan sequence was conducted to determine pretreatment ⁸⁹Zr-bevacizumab tracer uptake (Supplementary Fig. S1). Daily everolimus treatment [10 mg/kg intraperitoneal (i.p.) administration] was started immediately after completing the baseline scan sequence and continued for 14 days. On day 9, during everolimus treatment, a second 6-day scan sequence was initiated after ⁸⁹Zr-bevacizumab injection. Animals that received everolimus were sacrificed immediately after acquiring the posttreatment scans.

To allow comparison between treated versus untreated tumors, a separate group of mice (n = 5, control group) was sacrificed after the baseline scans. Mice xenografted with A2780huC cells develop rapidly growing tumors, with a doubling time of 3 to 6 days (16). Therefore, control animals cannot be maintained for the same duration as the treatment group, as tumors will grow beyond tolerable sizes.

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MicroPET imaging

Bevacizumab conjugation and labeling were conducted as described previously (15). Briefly, quality controls included size-exclusion high-performance liquid chromatography (SE-HPLC), trichloroacetic acid (TCA) precipitation, and immunoreactivity analysis. Radiochemical purity of all injected tracers was more than 95%. ⁸⁹Zr-bevacizumab (±5 MBq, ±30 μg) was injected into the penile vein. Animals were imaged using a microPET Focus 220 rodent scanner (CTI/Siemens). Static images were obtained (15–45 minutes acquisition) immediately after tracer injection and at 144 hours after tracer injection. In earlier tracer validation studies, it was shown that the ⁸⁹Zr-bevacizumab tumor uptake was significantly higher as compared with ⁸⁹Zr-IgG uptake, showing target-driven, tumor-specific binding and accumulation of the tracer (15, 16). ⁸⁹Zr-bevacizumab tumor uptake is optimal in this animal model at 144 hours after tracer injection (15, 16). The tracer dose was also based on these tracer validation studies (25). At this tracer dose, any retained cold antibody from the first tracer injection is not expected to influence tumor uptake at the second scanning period. Images were analyzed and quantified using AMIDE software [version 0.9.1, Stanford University (Stanford, CA); ref. 26]. Imaging data are presented as the mean standardized uptake value (SUVmean; ref. 17).

Biodistribution

Organs and tissues were excised, rinsed of residual blood, and weighed. Tissues were counted for radioactivity in a calibrated well-type LKB-1282-Compu-gamma system (LKB Wallac), together with reference tracer material to correct for physical decay. Ex vivo tissue activity is expressed as the percentage of the injected dose per gram of tissue (%ID/g). Harvested tumors were divided, and partly paraffin-embedded and partly frozen at −80°C for further ex vivo analysis.

Immunohistochemistry

Slices (3 μm thick) were cut from paraffin-embedded tumor sections using a microtome and placed on 3-aminopropyltriethoxysilane–coated glass slides. Heat-induced antigen retrieval was conducted in 10 mmol/L citrate buffer (S6, p-S6) or Tris/EDTA (Ki67) using a 400 W rotary microwave, or by using Proteinase K (Von Willebrand factor (vWF)) at room temperature. Endogenous peroxidase was blocked by 30 minutes incubation with 0.3% H₂O₂ in PBS. Endogenous avidin/biotin activity was blocked using a commercially available blocking kit (Vector Laboratories). Slides were incubated with primary antibodies detecting human S6 and p-S6 (Cell Signaling, #2217 and #2211) and Ki67 (Dako, 1:350). In addition, slides were stained with anti-vWF antibody (Dako, 1:250) to determine the mean vascular density (MVD; ref. 17). Staining was visualized after incubation with biotinylated or peroxidase-bound secondary antibodies (Dako) using, when necessary, the streptavidin–biotin/HRP complex (Dako) and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich). Hematoxylin counterstaining was applied routinely and hematoxylin and eosin (H&E) staining served to analyze tissue viability and morphology.
Stainings were quantified by evaluation of 10 high-power fields (HPF; ×400 magnification) using a calibrated grid, counting positive and negative cells (S6 and p-S6, cytoplasmic staining) or nuclei (Ki67). Data are expressed as the percentage of positive cells. MVD was determined by counting and averaging the number of vessels within 10 HPFs. Photographs were acquired by digitalized scanning of slides using the NanoZoomer 2.0-HT multi-slide scanner (Hamamatsu). To calculate tumor viability, acquired images from H&E-stained slides were analyzed using NanoZoomer Digital Pathology (NDP) viewer software (Hamamatsu). Using the freehand surface area tool, areas with nonviable tumor tissue were delineated within fixed-size squares overlaying the surface area of the slide.

**Statistical analyses**

Data are presented as mean ± SD. For in vitro experiments, different experimental conditions were compared using unpaired Student t tests. Comparison of uptake before and after everolimus treatment was conducted using the paired t test, whereas unpaired testing was used for ex vivo analyses (comparing control vs. treatment group data). Correlations were determined by Pearson correlation coefficient. Statistical analyses were conducted using Prism v.5 (GraphPad). A P value of less than 0.05 (two-tailed) was considered significant.

**Results**

**mTOR inhibition by everolimus reduces ovarian cancer VEGF-A secretion in vitro**

Target inhibition by everolimus was shown by reduced phosphorylation of p70S6K, S6, and 4E-BP1 in all cell lines tested (Fig. 1A). mTOR inhibition occurred within 2 hours upon everolimus exposure (Fig. 1B).

Everolimus reduced VEGF-A secretion by all cell lines (P < 0.05 at all concentrations used, compared with untreated controls; Fig. 2). The maximum effect was achieved at relatively low concentrations of everolimus (10 nmol/L). After 24 hours of drug exposure, VEGF-A levels in the culture supernatant were reduced by 57% ± 7% in A2780, 42% ± 15% in SKOV-3, and 38% ± 3% in TOV-21G cells as compared with untreated cells. Cell counting experiments revealed no effect on cell number or viability after 24 hours everolimus incubation.

**Everolimus reduces 89Zr-bevacizumab tumor uptake in xenografted mice**

89Zr-bevacizumab tracer uptake was homogeneously present within the tumors analyzed at each timepoint based on microPET scans. Everolimus decreased 89Zr-bevacizumab tumor uptake compared with pretreatment (baseline) scans in all animals. Figure 3A shows representative PET images. In pretreatment scans, the SUV mean was 2.9 ± 0.2.

![Figure 1](#)

Figure 1. A, A2780, SKOV-3, and TOV-21G cells were treated for 24 hours with everolimus at the indicated concentrations. Western blotting showed that phosphorylation of downstream mTOR target proteins S6, p70S6K, and 4E-BP1 was effectively reduced in all cell lines at all concentrations used. The upper band in the (p-) p70S6K blot is a consequence of analogous binding to p85S6K, which is another S6K splice variant. The α, β, and γ isoforms of 4E-BP1 reflect differential phosphorylation, with the γ-isofrm being most extensively phosphorylated. β-Actin was used as a loading control. B, A2780 cells were serum-starved overnight before everolimus (100 nmol/L) was added to the culture medium. After 30 minutes of preincubation with everolimus, FCS was added for the indicated timepoints. As shown by the lack of p70S6K phosphorylation, mTOR is already inhibited shortly after drug exposure and inhibition persisted throughout the whole incubation period.
compared with 2.3 ± 0.2 in posttreatment scans after 2 weeks of everolimus treatment (21.7% ± 4.0% decrease in tracer uptake, \( P = 0.0005 \); Fig. 3B).

Analogous, \textit{ex vivo} biodistribution experiments showed a lower \(^{89}\)Zr-bevacizumab tumor uptake in everolimus-treated animals versus control animals (7.8 ± 0.8% ID/g vs. 14.0 ± 1.7% ID/g, \( P < 0.01 \); Fig. 3C). These results correlated with microPET quantification data (\( R^2 = 0.93, P < 0.0001 \)). No differences in \(^{89}\)Zr-bevacizumab uptake were observed for nontumor tissues between treated and untreated animals (Fig. 3D).

During the 14-day treatment period, tumor volumes in mice receiving everolimus increased to a maximum of 135% ± 17% as compared with the start of treatment (Fig. 4). Tumor growth during everolimus treatment was slower as compared with historical controls (untreated A2780\textsubscript{Luc} xenograft-bearing mice), which showed a similar increase in tumor volume already at day 3 after baseline scans (17).

**Decreased \(^{89}\)Zr-bevacizumab tumor uptake corresponds with reduced tumor VEGF-A levels and reduced vascularity after everolimus treatment**

Everolimus treatment resulted in effective target inhibition as the expression of phosphorylated S6 was clearly reduced in treated tumors versus tumors obtained from control animals after baseline scanning, whereas total S6 expression was not affected (Fig. 5A). Morphologic analysis of H&E-stained slides revealed no difference in tumor viability between treated and control tumors (Fig. 5B). After 2 weeks of everolimus treatment, the Ki67-based proliferation index was lower in treated (30.21% ± 1.76%) as compared with control tumors (50.86% ± 1.46%, \( P < 0.0001 \); Fig. 5C).

VEGF-A protein expression was lower in everolimus-treated versus control tumors (0.33 ± 0.02 vs. 0.50 ± 0.16 pg/µg total protein, \( P = 0.04 \); Fig. 5D). In addition, everolimus treatment reduced tumor vascularity, as the MVD averaged 4.1 ± 1.4 in treated versus 7.8 ± 1.4 vessels per HPF (\( P < 0.01 \)) in control mice (Fig. 5E). VEGF-A protein levels correlated with microPET quantification data (\( R^2 = 0.43, P < 0.05 \)) and \textit{ex vivo} biodistribution data (\( R^2 = 0.44, P < 0.05 \)).

**Discussion**

This study shows that \textit{in vivo} serial \(^{89}\)Zr-bevacizumab PET imaging can visualize reduced tumor VEGF-A levels upon treatment with everolimus in an ovarian cancer xenograft model.

The relatively modest activity of temsirolimus observed in patients with ovarian cancer underscores the need for patient selection. Responses limited to a subset of patients with ovarian cancer have also been observed with VEGF(R)-targeted antiangiogenic drugs, as well as several other targeted agents, and could result from marked molecular heterogeneity, which is characteristic to ovarian cancer (27, 28). The discovery of biomarkers for (early) response prediction might permit the selection of patients that are likely to benefit from single-agent mTOR inhibition or mTOR inhibitor-based combination regimens. Several proteins involved in mTOR signaling have been proposed as suitable biomarkers for response. Phosphorylation of mTOR in ovarian cancer specimens is a poor indicator of its kinase activity (5). Phosphorylation of downstream target proteins (e.g., p70S6K, S6, and 4E-BP1) is a better read-out for mTOR activity and has been used to check for effective drug delivery and optimal biologic dosing in phase I clinical trials with everolimus (20, 29). Using these protein expression levels as biomarkers for response is hampered by the need for (repeat) tumor tissue acquisition, or the use of surrogate tissues, such as peripheral blood mononuclear cells, skin, or hair follicles. Instability of phosphorylated proteins further challenges the reliability (and reproducibility) of such analyses (30).

\textit{In vivo} molecular imaging provides an attractive alternative. It allows for whole-body, noninvasive monitoring of tumor biology, and changes herein resulting from drug administration. These techniques are being explored as tools for patient selection for several molecular-targeted drugs, including mTOR inhibitors. Thus far, \[^{18}F\]fluorodeoxy-2-deoxy-\(\alpha\)-glucose PET (FDG-PET) measuring tumor glucose metabolism has received most attention (31, 32). Inhibition of mTOR does indeed reduce glucose uptake by reducing glucose transporter 1 (GLUT1) expression and/or hexokinase activity (33). However, changes in FDG-PET
everolimus on VEGF-A levels in xenografted ovarian cancers with 89Zr-bevacizumab PET. Everolimus treatment resulted in reduced 89Zr-bevacizumab tumor uptake, which coincided with delayed tumor growth, lower tumor VEGF-A protein expression, and reduced MVD.

Accumulation of radiolabeled bevacizumab results from its interaction with larger human VEGF-A isoforms bound to (tumor) cell surfaces and extracellular matrix components (34). For VEGF-A165, the most abundant isoform, equilibrium is thought to exist between bound and freely diffusible fractions due to weak binding properties. 89Zr-bevacizumab PET thus has the potential of not only visualizing presence but also activity of tumor-associated VEGF-A. This is supported by the finding that there was a 48% reduction in tumor vascularity (MVD) after everolimus treatment, showing an antiangiogenic response. These findings are of interest to evaluate in the clinic. 89Zr-bevacizumab PET is already known to show excellent tracer uptake in tumor lesions of patients with renal cell cancer and is currently tested as an early biomarker for response to everolimus in 2 clinical trials (Everbilimgage, NCT01028638; NETPET, NCT01338090; ref. 35). Moreover, 89Zr-bevacizumab PET might provide more insight in differential effects of antiangiogenic drugs on individual tumor lesions within a patient.

It is of interest that we observed a homogeneous decrease in tracer uptake after everolimus treatment based on microPET scans. This was also observed previously with the HSP90 inhibitor NVP-AUY922 (16). This differs from previous findings with the VEGFR-targeted TKI sunitinib, which acts at the level of the endothelial cells. 89Zr-ranibizumab, the radiolabeled Fab-fragment derived from bevacizumab, was used to visualize the effect of sunitinib treatment on tumor VEGF-A levels in an ovarian cancer xenograft model (17). Reduction of tracer uptake was more pronounced in the tumor center versus the rim of the tumor.
Increased invasiveness was observed in xenograft models treated with sunitinib, and it was speculated that hypoxia (and consequently HIF activity) drives this adaptive response (36). Lack of differential tracer uptake between tumor center and periphery observed with mTOR or HSP90 inhibitors might reflect their potential to inhibit HIF activity, through reducing its translation or stability.

Our results provide a proof-of-principle of using $^{89}$Zr-bevacizumab PET to monitor reduction of tumor VEGF-A levels in response to antiangiogenic treatment with everolimus. The $^{89}$Zr-bevacizumab PET provides a novel tool for noninvasive monitoring of antiangiogenic effects upon mTOR inhibitor therapy. It provides an interesting candidate biomarker in cancers in which angiogenesis inhibition is thought to underlie treatment efficacy, such as ovarian cancer. Our results support the evaluation of $^{89}$Zr-bevacizumab PET to measure tumor VEGF-A levels as an early biomarker for antiangiogenic efficacy of mTOR inhibitor therapy in clinical studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Figure 5.** A, S6 expression was homogeneously present in more than 90% of tumor cells, whereas p-S6 was heterogeneously expressed. Everolimus treatment did not change total S6 expression, whereas p-S6 expression was significantly reduced in treated versus control tumors ($P < 0.001$). There was a marked decrease in the ratio between p-S6/S6, which was determined for each individual tumor. B, tumor viability is expressed as the percentage of viable tumor tissue within the total surface area analyzed. No significant differences in tumor viability were observed between treated and control tumors. C, the proliferation index is expressed as the percentage of nuclei positive for Ki67. Compared with control tumors, everolimus treatment reduced the proliferating fraction by 41% ($P < 0.001$). D, VEGF-A levels determined in whole-tumor lysates show reduced VEGF-A in everolimus-treated versus control tumors ($P = 0.04$). VEGF-A levels are expressed relative to the total protein content of each lysate, to correct for loading differences. E, MVD, presented as the average number of vWF-positive vessels per HPF, was reduced in treated versus control tumors (48% decrease, $P < 0.01$). A representative picture is included that shows an angiogenic area in a control tumor, showing specific staining of tumor vasculature.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.R.M. van der Bilt, A.G.T. Terwisscha van Scheltinga, E.G.E. de Vries, A.K.L. Reynolds


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