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Demonstration of highly specific toxicity of the α-emitting radioimmunoconjugate ^{211}At-rituximab against non-Hodgkin's lymphoma cells

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Summary The ability of an α-emitter conjugated to a chimaeric anti-CD20 monoclonal antibody to kill selectively human B-lymphoma cells in vitro is reported. Two B-lymphoma cell lines RAEL and K422, and normal haematopoietic progenitor cells from human bone marrow aspirates were incubated with ^{211}At-rituximab (Rituxan® or MabThera™) and plated in clonogenic assays for survival analyses. Following 1 h incubation with ^{211}At-rituximab, in concentrations which gave an initial activity of 50 kBq ml^{-1}, a high tumour cell to normal bone marrow cell toxicity ratio was obtained; 4.1 to 1.0 log cell kill. Biodistribution studies of ^{211}At-rituximab in Balb/c mice showed similar stability as that of the iodinated analogue. The data indicate that testing of ^{211}At-rituximab in human patients is warranted. © 2000 Cancer Research Campaign

Keywords: clonogenic survival; lymphoma cells; bone marrow; α-emitter; radioimmunoconjugate; rituximab

Most patients with relapsed non-Hodgkin's lymphoma (NHL) are incurable by current conventional treatment. High-dose chemotherapy with stem cell support is effective only to patients with chemosensitive disease (Armitage, 1993). Hence, new treatment modalities are needed. Various immunotherapeutic approaches, including the use of native monoclonal antibodies (MoAbs), immunotoxins and radioimmunoconjugates, have been investigated (for review see Multani and Grossbard, 1998). MoAbs against the CD20 antigen given to patients with relapsed B-cell NHL yielded response rates of about 30–50% (McLaughlin et al, 1998). When conjugated to the β-emitting isotopes ^{131}I (Press et al, 1995; Kaminski et al, 1996) or ^{90}Y (Knox et al, 1996), responses in up to 86% were seen (Multani and Grossbard, 1998; Liu et al, 1998). Radioimmunotherapy is dose-limited mainly by haematological toxicity, unless stem cell support is given.

The α-emitter ^{211}At has several favourable physical properties compared to the currently used β-emitters. Thus the half-life ($t_{1/2}$) of ^{211}At is only 7.2 h, whereas ^{131}I and ^{90}Y have half-lives of 8.0 days and 64 h, respectively. Alpha particles from ^{211}At have an average energy of 6.8 MeV and their range in soft tissue is only 55–80 μm, i.e. a few cell diameters. Because of the low range of the α-radiation in tissues, need for patient shielding is greatly reduced, allowing α-radioimmunotherapy to be given on an out-patient basis (Larsen et al, 1999). Moreover, the high LET (linear energy transfer) of ^{211}At, about 97 keV μm^{-1}, is close to the optimum value for high RBE (relative biological effectiveness) (Brown, 1986). Alpha emitters are also generally associated with low oxygen enhancement ratio and virtual absence of dose–rate effects (Barendsen et al, 1965; Hall, 1994). The short range of the α-emitters implies that the radiation dose is delivered to the cell or within its close vicinity. This fact, together with the targeting ability of the MoAb, makes α-particle-emitting immunoconjugates attractive for radioimmunotherapy against dispersed cells and micrometastases.

The present study was performed to explore the ability of ^{211}At-rituximab to selectively inactivate NHL tumour cells with acceptable bone marrow damage.

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MATERIALS AND METHODS

Haematopoietic cells and lymphoma cell lines

Fresh mononuclear bone marrow (BM) cells from healthy volunteers were used. BM may contain various amounts of B-lymphocytes (CD20$^+$), from < 1% up to 15–20%. Peripheral blood progenitor cells (PBPC) were harvested in the recovery phase following chemotherapy and G-CSF (in patients with other diagnoses than NHL), and enriched CD34$^+$ haematopoietic cells (from PBPC) were used. Two B-cell NHL cell lines in exponential growth, RAEL (high-grade) and K422 (low-grade) (Dyer et al, 1990), expressing the CD20 antigen, were studied.

Monoclonal antibody

A chimaeric anti-CD20 MoAb with murine variable regions and human IgG_1-kappa constant regions, IDEC-C2B8 or rituximab, was used. This MoAb is marketed by IDEC Pharmaceutical Corporation, San Diego CA and by Genentech Inc, San Francisco CA in the USA under the name of Rituxan®, and in Europe by Hoffmann-La Roche Ltd, Basel, Switzerland under the name MabThera™. The CD20 antigen is expressed at high density on malignant B-cells as well as normal B-lymphocytes, but not on early B-cells and stem cells. Within the time-frame of ^{211}At decay, the CD20 antigen is virtually stable on the cell surfaces (Reff et al, 1994).

Radionuclides and labelling

Astatine-211 was produced in a cyclotron at the Department of Physics, University of Oslo (Larsen et al, 1994 *a*) using the $^{209}Bi(\alpha, 2n)\ ^{211}At$ reaction. Commercially available ^{125}I (New England Nuclear, Billerica MA, USA) was used for the iodine labelling. Rituximab was radiolabelled with ^{211}At and ^{125}I and purified as previously described for other proteins (Larsen et al, 1994 *b*). The resulting radioimmunoconjugates, ^{211}At-rituximab and ^{125}I-rituximab, were sterile-filtered prior to the biological experiments, using a Millex-GV 0.22 µm filter (Millipore, Bedford MA, USA).

In the present experiments the specific activity varied from 10–75 MBq mg^{-1}. The specific binding fraction of ^{211}At-labelled rituximab was determined in a one-point assay. Cell-bound radioimmunoconjugate was measured in triplicate after incubation at room temperature for 1–2 h, using approximately 10^7 cells in 0.3 ml medium. An average of 36.4% of the labelled antibody (range 17.3–54.1%) bound to antigen-positive RAEL cells, whereas non-specific binding of ≤ 1.8% was measured on the antigen-negative osteosarcoma cell line OHS. A binding fraction of 36% in the one-point assay corresponds to an immunoreactive fraction at infinite antigen excess (Lindmo et al, 1984) of about 50–60%, as verified experimentally (data not shown).

Cell killing activity of ^{211}At-rituximab

Different amounts of ^{211}At-rituximab were added to a fixed concentration of 10^6 cells ml^{-1}. Initial radioactivity concentrations (kBq ml^{-1}) in the test tubes were counted in a LKB Wallac 1277 Gammamaster gammacounter (Wallac Oy, Turku, Finland). Two experimental series with different incubation time were performed: A 1 h incubation series to study the efficacy of cell-bound radioimmunoconjugate; and a 4 h incubation series to simulate the prolonged circulation in vivo of IgG-type radioimmunoconjugates. Four hours are similar to the effective half-life of the radiopharmaceutical in the body, as indicated in mice experiments. After the incubation, under soft stirring for 1 or 4 h, the cells were washed in culture medium and centrifuged three times. Then survival was measured in clonogenic assays, and compared to that observed in the absence of radioactivity.

The number of clonogenic tumour cells remaining after exposure to ^{211}At-rituximab was assessed by a modified version (Kvalheim et al, 1987) of the Courtenay and Mills soft agar clonogenic assay (Courtenay and Mills, 1978). For evaluation of toxicity on haematopoietic cells the CFU-c (colony forming unit cell) assay with METHOCULT H4433 medium (Stem Cell Technologies Inc, Vancouver, Canada) was used. The toxicity of the labelled MoAbs was also tested on more primitive (stem cell-near) haematopoietic cells, using the LTC-IC (long-term culture initiating cell) assay (Eaves et al, 1991). Briefly, the cells were seeded on a pre-irradiated (20 Gy) human bone marrow stroma mono-layer in the medium MYELOCULT H5100 (Stem Cell Technologies Inc), supplemented with 50 µM hydrocortisone, and cultured for 5 weeks before secondary culture in CFU-c assay.

Therapeutic gain

The relationship between the initial concentration of radioactivity (kBq ml^{-1}) and clonogenic survival was, in the range tested, best fitted by quadratic regression. The concentration of ^{211}At-rituximab giving a survival of 10% or 37% (A_{10} or A_{37}) was calculated from the survival curves. A therapeutic gain factor (TGF) for tumour cells relative to haematopoietic cells exposed to ^{211}At-rituximab, was calculated using the following equation:

$$TGF_{37} = A_{37,\ BM}/A_{37,tumour}$$

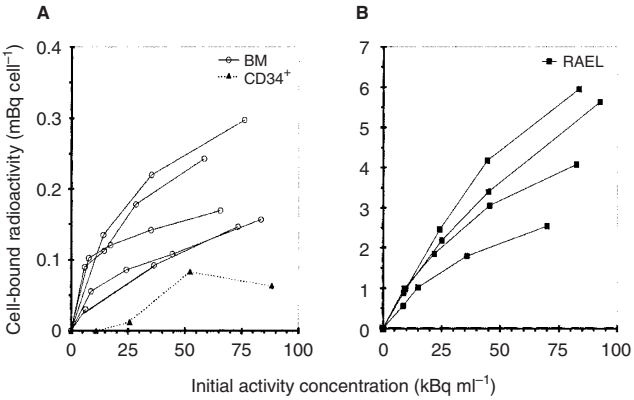

Figure 1 Cell bound ^{211}At-rituximab related to the initial activity concentration. Radioactivity (mBq cell^{-1}) bound to normal bone marrow cells (BM) and to CD34$^+$-selected haematopoietic cells (**A**) and to the lymphoma cell line RAEL (**B**). Note differences in scale on the y-axis

Biodistribution

Biodistribution was studied in Balb/c mice. Astatine-211 and ^{125}I were examined in a 'paired label' fashion where 30 kBq of ^{125}I-labelled and 120 kBq of ^{211}At-labelled rituximab in 100 µl were co-injected into the tail vein of each animal. Approximately 4 µg rituximab were injected per mouse. At various time-points three mice were sacrificed, the organs were dissected out and weighed, the radioactivity contents were measured and the percentage of injected dose per g of tissue was determined.

Statistical analyses and curve estimations

SPSS 8.0, EXCEL 97 for Windows, and SigmaPlot (Scientific Graphic Software, version 2, Jandel Corporation) were used in the statistical analyses, plots and graphs. The input values for the survival curves were calculated by the mean $\log_{10}$ anti-log of surviving fractions for separate doses in the individual experiments (presented in the Figures). Survival curves were fitted to the data using quadratic regression of $\log_{10}$ survival. The 95% confidence intervals (CI) of the survival curves were calculated using $\log_{10}$ of the 95% CI from each point on the survival curves.

RESULTS

Cell-bound radioactivity

Cell-bound radioactivity (mBq cell^{-1}), measured after 1 h incubation, is presented in Figure 1A for normal BM and CD34$^+$ cells and in Figure 1B for the RAEL cells. It is seen that, as expected, much more radioactivity was bound to the tumour cells than to the normal BM cells.

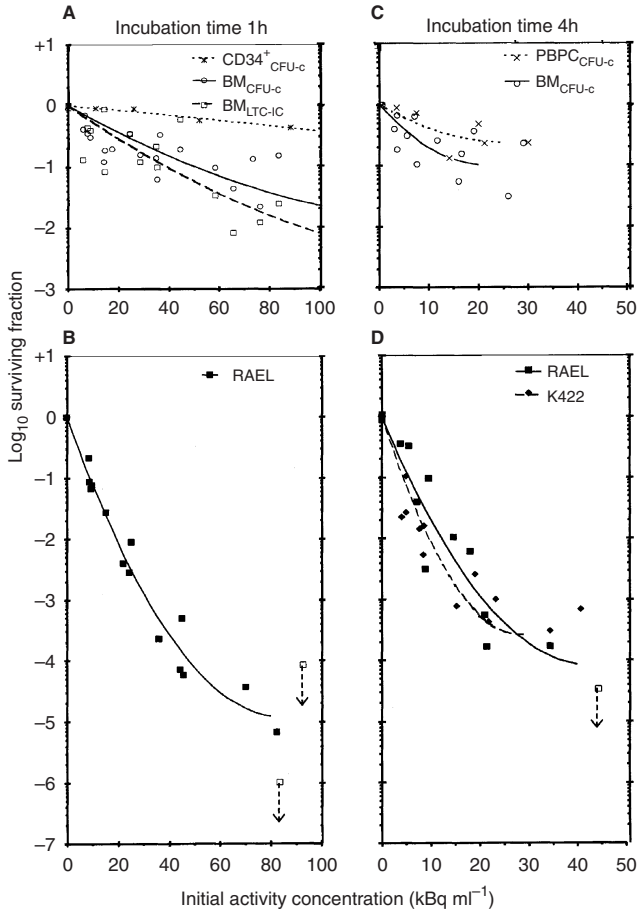

Figure 2 Clonogenic survival of haematopoietic cells and lymphoma cells related to initial concentration of radioactivity (kBq ml^{-1}) of ^{211}At-rituximab. Incubation times were 1 h (**A** and **B**) and 4 h (**C** and **D**). The logarithmic mean surviving fractions from each experiment (3–9 parallels at each point), and the quadratic regression lines are marked. The open symbols with arrows (**B** and **D**) represent points where no colonies were observed at the highest seeded cell concentration. (**A**) BM, evaluated in both CFU-c and LTC-IC assays, as well as CD34+ cells plated in CFU-c assay, are compared, while in (**B**) the surviving fractions of RAEL are shown. (**C**) Survival of BM and PBPC following 4 h incubation and (**D**) the two tumour cell lines, RAEL and K422.

Survival of lymphoma cells and haematopoietic cells following exposure to ^{211}At-rituximab

Clonogenic survival vs initial activity concentration of ^{211}At-rituximab is shown in Figure 2. The data in Figure 2A represent experiments with BM cells from five different donors tested in CFU-c, four of them also examined in LTC-IC assays. In addition, one experiment with enriched CD34+ cells is included in the figure. In Figure 2B four individual experiments with RAEL cells are shown. Comparison of Figure 2A and 2B (1 h measurements) demonstrates a highly significant, favourable survival of the BM cells compared to the malignant RAEL cells. From the survival curves the following mean A_{10} values were estimated: 50.0 kBq ml^{-1} for BM_{CFU-c}, 39.1 for BM_{LTC-IC}, 203.5 for $CD34^{+}_{CFU-c}$ and 9.0 for RAEL. An initial activity concentration of 50 kBq ml^{-1} yielded corresponding log cell kill values of 1.00, 1.24, 0.20 and 4.13. In Table 1 the values are given with 95% CI.

BM cells tested in LTC-IC were separated from cells in standard CFU-c assay just before plating. Hence, these stem cells were exposed to the same crossfire irradiation from B-cells in the pellets before seeding. The experiments showed a tendency of higher toxicity on BM evaluated in LTC-IC as compared to CFU-c assay, but the differences were not significant, $P = 0.36$ (paired samples t-test, two-tailed). In contrast, the toxicity towards CD34+ cells was lower for the tested initial concentrations of activity (Figure 2A).

In Figure 2C and 2D the results of 4 h measurements are shown. Since 4 h incubation may reflect a more realistic time schedule with regard to the effective half-life of ^{211}At-rituximab administered to patients, the study was extended with these series. The data in Figure 2C and 2D represent three individual experiments for each tumour cell line, BM from three different donors and two PBPC products. Again the ^{211}At-rituximab was found to be far more toxic to the tumour cells than to the haematopoietic cells. However, neither between BM and PBPC nor between the two tumour cell lines were significant differences found. Calculations from the survival curve parameters give the following mean A_{10} values (kBq ml^{-1}): 22.8 for BM, 5.6 for RAEL and 4.3 for K422. Such low survival level was not observed for PBPC in the given range of initial activity concentrations. At an initial activity concentration of 25 kBq ml^{-1}, the mean log cell kills were 1.01 for BM_{CFU-c}, 0.61 for $PBPC_{CFU-c}$, 3.39 and 3.54 for RAEL and K422, respectively (Table 1).

The amounts of radioactivity added in this study correspond to a rituximab concentration of less than 8 μg ml^{-1}. In a control experiment with unlabelled rituximab at similar concentrations as used with the radioimmunoconjugate, no significant effect on survival, neither of the RAEL nor the BM cells, was observed (data not shown). This indicates that the radioisotope was essential for the effectiveness of ^{211}At-rituximab in the present experiments. The concentrations used represent less than 3.5% of the serum concentration obtained in clinical trails using therapeutic doses of native rituximab (McLaughlin et al, 1998).

Table 1 A_{10} values[a] and log cell kills[a] following exposure to ^{211}At-rituximab

Cells	BM_{CFU-c}	BM_{LTC-IC}	$CD34^{+}_{CFU-c}$	$PBPC_{CFU-c}$	RAEL	K422
Incubation time – 1 h						
A_{10} (kBq ml^{-1})	50.0	39.1	203.5	–	9.0	–
	(45.4–56.4)	(27.9, 45.4)	(167.1, –)		(7.7, 9.8)	
Log cell kill	1.00	1.24	0.20	–	4.13	–
at 50 kBq ml^{-1}[b]	(0.91–1.10)	(1.09, 1.61)	(0.12, 0.28)		(3.95–4.50)	
Incubation time – 4 h						
A_{10} (kBq ml^{-1})	22.8	–	–	–	5.6	4.3
	(12.6, –)				(5.5–6.0)	(4.1–4.6)
Log cell kill	1.01	–	–	0.61	3.39	3.54
at 25 kBq ml^{-1}[b]	(0.86–1.14)			(0.44–0.71)	(3.18–3.96)	(3.38–3.70)

[a]Mean (95%CI); [b]initial activity concentration

Table 2 Therapeutic gain factor at 37% cell survival (TGF_{37})[a] following exposure to ^{211}At-rituximab

	RAEL to				K422 to	
Incubation time	BM_{CFU-c}	BM_{LTC-IC}	$CD34^{+}_{CFU-c}$	$PBPC_{CFU-c}$	BM_{CFU-c}	$PBPC_{CFU-c}$
1 h	5.2 ± 0.7	4.2 ± 1.2	26.8 ± 3.9	–	–	–
4 h	2.3 ± 0.5	–	–	5.0 ± 2.8	3.0 ± 0.7	6.5 ± 3.7

[a]Mean ± SD

Table 3 Biodistribution[a] of ^{211}At- and ^{125}I-rituximab in mice

	1 h		3.5 h		10.75 h		23.5 h	
Tissue	^{211}At-rituximab	^{125}I-rituximab	^{211}At-rituximab	^{125}I-rituximab	^{211}At-rituximab	^{125}I-rituximab	^{211}At-rituximab	^{125}I-rituximab
Blood	36.2 ± 2.9	37.9 ± 2.1	32.9 ± 1.9	32.3 ± 2.0	27.1 ± 1.2	28.5 ± 0.6	17.4 ± 1.4	20.1 ± 2.0
Lung	15.5 ± 3.6	15.7 ± 3.8	13.4 ± 1.5	12.6 ± 2.0	11.6 ± 1.3	11.1 ± 1.2	8.5 ± 1.9	8.7 ± 1.8
Liver	11.5 ± 1.5	13.0 ± 1.7	9.5 ± 1.0	10.5 ± 0.8	6.7 ± 0.7	8.2 ± 1.2	4.4 ± 1.2	4.2 ± 3.4
Heart	10.5 ± 0.8	10.1 ± 0.8	10.2 ± 0.9	11.2 ± 1.2	8.9 ± 1.3	8.4 ± 0.5	6.8 ± 0.9	6.9 ± 0.9
Spleen	8.1 ± 2.7	8.7 ± 1.9	7.9 ± 0.9	7.6 ± 0.7	7.3 ± 0.6	7.2 ± 0.2	4.6 ± 0.8	5.0 ± 0.6
Kidney	7.9 ± 0.8	9.1 ± 0.8	8.5 ± 1.1	9.0 ± 1.2	6.5 ± 1.3	7.7 ± 0.9	4.1 ± 0.1	5.2 ± 0.6
Muscle	1.0 ± 0.4	1.2 ± 0.3	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.5 ± 0.3	1.8 ± 0.3
Bone	2.8 ± 0.7	2.9 ± 0.3	2.8 ± 0.5	2.9 ± 0.1	3.0 ± 0.2	3.2 ± 0.2	2.0 ± 0.4	2.9 ± 0.2

[a]Values expressed as % of i.v. injected dose of ^{211}At- and ^{125}I-rituximab per gram tissue, mean ± SD

Therapeutic gain

Both 1 h and 4 h incubation with ^{211}At-rituximab gave favourable therapeutic windows between tumour cells and haematopoietic cells. The average TGF_{37} ± SD, is presented in Table 2. In the 1 h series TGF_{37} between RAEL and BM_{CFU-c} was 5.2 in average. TGF_{37} for tumour cells vs BM_{CFU-c} in the 4 h series were 2.3 for RAEL and 3.0 for K422.

Biodistribution in mice

Table 3 presents the tissue distribution of ^{211}At-rituximab and ^{125}I-rituximab in eight organs of the mice, within a time-frame of 1–23.5 h. The biodistribution of ^{211}At-rituximab was similar compared to that of ^{125}I-rituximab, and revealed the general characteristics of IgG radiolabelled MoAbs, i.e. high initial activity levels in blood and blood-rich tissues.

DISCUSSION

The reported experiments were designed to assess targeted α-emitter exposure to dispersed B-lymphoma cells and haematopoietic progenitor cells.

The normal bone marrow cells generally showed a low sensitivity following exposure to ^{211}At-rituximab. BM contains various amounts of B-lymphocytes, ranging from < 1% up to 20%, which may explain the considerable variation in cell-bound activity observed in different experiments. The difference in toxicity on the BM cells, reflected in the survival curve (Figures 2A and 2C), could possibly be explained by crossfire irradiation of the antigen-negative clonogenic cells from B-cells in the pellets before seeding. This is further supported by the very low toxicity observed with $CD34^{+}$-enriched cells, which had low binding of ^{211}At-rituximab, due to the virtual absence of B-cells in this cell population. Furthermore, PBPC products, which contain about 0.5–1% B-cells, also showed a trend towards lower cytotoxicity from the radioimmunoconjugate compared to BM, although the difference was not statistically significant. Neither was the survival significantly different between BM cells tested concurrently in the CFU-c and the LTC-IC assay.

Astatine-211-labelled rituximab was highly cytotoxic to both high- and low-grade NHL cells when measured after 4 h incubation, and no significant difference in survival between the two cell lines was found (Figure 2D).

In our experiments, A_{37} following 1 h incubation was 3.8 and 19.5 kBq ml^{-1} for RAEL and BM_{CFU-c}, respectively, whereas the calculated value for $CD34^{+}$ cells was as high as 100.9 kBq ml^{-1}. Hence, A_{37} for $CD34^{+}$ cells was almost identical to the values previously reported for human cell lines exposed to non-specific ^{211}At-BSA (bovine serum albumin) (Larsen et al, 1994*a*).

The biodistribution profile indicated that the quality of the immunoconjugate had not been significantly reduced by labelling with ^{211}At and also that the stability of the conjugate in vivo was adequate. As with ^{131}I-labeled compounds a blocking agent should be used for therapeutic purposes to minimize radiation dose to the thyroid for ^{211}At (Larsen et al, 1998).

The current study confirms previous reports indicating that α-emitting immunoconjugates may have selective anti-tumour effect. One study by Harrison and Royle (1987) used ^{211}At conjugated to a T-cell antibody (OX7) to treat a T-cell lymphoma in mice and increased the median survival time of mice and probably 'cured' more than half of the animals (Harrison and Royle, 1987). Later Behr and colleagues (1999) compared the therapeutic efficacy and toxicity of the α-emitter ^{213}Bi with that of the β-emitter ^{90}Y, linked to a monovalent Fab′ fragment, in a human colonic cancer xenograft model in nude mice (Behr et al, 1999). They concluded that radioimmunotherapy with α-emitters may be therapeutically more effective than conventional β-emitters. Recently pharmacokinetics and dosimetry of a ^{213}Bi-labelled anti-CD33-antibody in patients with leukaemia was reported (Sgouros et al, 1999).

Clinical studies with radioimmunoconjugates (^{131}I and ^{90}Y) in lymphomas have mainly involved murine MoAbs. Using a chimeric MoAb like rituximab in radioimmunotherapy could

strongly reduce HAMA (human anti-mouse antibody) responses in patients. However, the prolonged biological half-life of the humanized MoAbs could cause problems with increased radiation dose to normal tissues, and hence a decreased therapeutic index, when isotopes with longer half-life (days) are utilized (Bruland, 1995; Multani and Grossbard, 1999). The use of a relatively short-lived isotope like ^{211}At will make those differences in biological half-life less relevant.

As pointed out, ^{211}At also has several advantages from a radiation-protection point of view. More than 99% of its radiation energy is from α-particles. The short half-life (7.2 h) could reduce the hospitalization time, and the short tissue-range of the α-particles (maximum 80 μm) would simplify the protection of the staff and environment (Larsen et al, 1999).

A BM progenitor cell survival of 5% should be sufficient for recovery without stem-cell support. This survival level of $BM_{CFU\text{-}e}$ was observed at an initial activity concentration of 70.3 kBq ml^{-1} following 1 h incubation. The corresponding log cell kill of RAEL was as high as 4.8. Consequently there may be a significant therapeutic window if non-myeloablative doses of ^{211}At-rituximab are explored clinically.

In conclusion, our study shows that rituximab labelled with ^{211}At exerts selective cytotoxicity to NHL tumour cells in vitro. The effect of the radioimmunoconjugate will now be investigated in patients with relapsed B-cell lymphomas.

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