Evaluation of Ricin A Chain-containing Immunotoxins Directed against the CD30 Antigen as Potential Reagents for the Treatment of Hodgkin's Disease

Andreas Engert, Francis Burrows, Wolfram Jung, Pier Luigi Tazzari, Harald Stein, Michael Pfreundschuh, Volker Diehl, and Philip Thorpe


ABSTRACT

Five monoclonal CD30 antibodies and two Fab' fragments were linked to deglycosylated ricin A chain (dgA), and their potential as immunotoxins for the treatment of Hodgkin's disease was evaluated. Cross-blocking experiments demonstrated that HRS-1, HRS-3, HRS-4, and Ber-H2 recognize the same epitope on the CD30 antigen and that Ki-1 binds to a different epitope. Scatchard analyses showed that HRS-3, HRS-4, and Ber-H2 bound strongly to L540 Hodgkin cells (Kd = 15, 7, and 14 nM, respectively), whereas HRS-1 and Ki-1 bound more weakly (Kd = 160 and 380 nM, respectively). The different affinities of the antibodies correlated closely with their cytotoxic potency as immunotoxins. HRS-3.dgA, HRS-4.dgA, and Ber-H2.dgA inhibited the protein synthesis of L540 cells by 50% at concentrations of 0.9-2.0 x 10^{-8} M, whereas HRS-1.dgA and Ki-1.dgA were about 100 times less potent with 50% inhibitory concentrations of 0.8-1.0 x 10^{-6} M. The most effective immunotoxins, HRS-3.dgA and HRS-4.dgA, were only 15 times less toxic than ricin itself. HRS-3 Fab'.dgA and HRS-4 Fab'.dgA were 7.8 and 3.3 times less potent than their IgG.dgA counterparts with 50% inhibitory concentrations of 7 x 10^{-6} M and 3 x 10^{-6} M, respectively. Staining of human tissues revealed an unexpected cross-reactivity of HRS-4 with pancreatic cells of malignant origin. HRS-1.dgA and Ki-1 showed very little cross-reactivity with any normal human tissues. It is concluded that HRS-3.dgA and HRS-3 Fab'.dgA are the immunotoxins of choice for in vitro therapy.

INTRODUCTION

Chemotherapy of Hodgkin's disease is undoubtedly one of the major breakthroughs in clinical oncology over the last 25 years. The introduction of the multiagent chemotherapy regimens such as mechloretamine-vincristine-procarbazine-prednisone (1) and Adriamycin-bleomycin-vinblastine-decarbazine (2) and the optimized use of radiation in early stages of the disease have improved the probability of curing these patients from less than 5% in 1963 to about 70% at the present time (3). Nevertheless, 30-50% of patients currently presenting with Hodgkin's disease at an advanced stage (IIIB, IVA, IVB) will still die from their disease (4). Those patients who are cured face the risk of developing a second malignancy which is relatively rare in Hodgkin's disease at an advanced stage (IIIB, IVA, IVB) will still die from their disease (4). The patients who are cured face the risk of developing a second malignancy which is reported to affect up to 19% within 15 years after treatment (5). New agents that are free from mutagenic side effects are therefore needed for the management of resistant disease.

One approach to preparing new, nonmutagenic reagents for the therapy of Hodgkin's disease would be to couple the ribosome damaging A chain of ricin or other toxins to antibodies directed against Hodgkin's cell-associated antigens. In several laboratories, ricin A chain has been linked to antibodies against tumor-associated antigens to form reagents ("immunotoxins") that are selectively toxic to malignant cells in vitro (reviewed in Refs. 6 and 7). However, in vivo studies in rodents and, more recently, in humans have given variable results. In rodents, good antitumor effects have generally been observed in leukemia and lymphoma models, whereas solid tumors appear to be less responsive (6, 7). In humans, the antitumor effects obtained in melanoma (8) and leukemia (9) patients have thus far been disappointing, whereas in patients with steroid-resistant graft-versus-host disease, remarkable benefit has been obtained (10).

We and others have identified and substantially overcome several problems that were reducing the antitumor activity of ricin A chain-containing immunotoxins in vivo. Rapid clearance of immunotoxins was occurring because liver parenchymal and nonparenchymal cells were recognizing mannoside and fucose residues on the A chain moiety (11); this was prevented by using chemically deglycosylated ricin A chain (dgA2) to form the immunotoxin (12). The immunotoxins were unstable in vivo because the disulfide linkages generated by the most widely used cross-linking agents, N-succinimidyl-3-(2-pyridyldithio)propionate and 2-iminothiolane, were prone to reduction; this was overcome by synthesizing new cross-linking agents, 4-succinimidoloxycarbonyl-α-methyl(2-pyridyldithio)toluene (13) and N-succinimidyl-3-(2-pyridyldithio)butyrate (14), which generate hindered disulfide linkages with improved stability. The size of immunotoxins prepared from intact IgG (M, 180,000) hampers their access to neoplastic cells in solid tumors; immunotoxins formed using the smaller Fab' fragment of antibody (M, 80,000) might home in larger amounts (15). CD22 immunotoxins incorporating these improvements have been developed for treating patients with B-cell tumors (16, 17) and are currently being tested in Phase I/II clinical trials.

Here we describe the preclinical evaluation of immunotoxins for the treatment of Hodgkin's disease. The five antibodies being considered recognize the CD30 antigen which is consistently expressed at high levels on Hodgkin and Reed-Sternberg cells (18, 19, 35) which are thought to be the malignant cells in this disease. We demonstrate that the HRS-3 antibody and its Fab' fragment form the most effective immunotoxins in vitro, show little cross-reaction with normal human tissues, and thus appear to be the reagents of choice for treating human disease.

MATERIALS AND METHODS

Materials. Tissue culture medium RPMI 1640 and fetal calf serum were purchased from Gibco-Biocult, Ltd. (Paisley, Scotland). Blue Sepharose CL-6B, Sepharose G-25 (fine grade), staphylococcal protein A-Sepharose, DEAE-Sepharose, and Sephacryl S-200HR were obtained from Pharmacia, Ltd. (Milton Keynes, England). Carrier-free 3Hl and L-[4,5-3H]leucine (TRK 170) were purchased from Amersham International (Amersham, England). Iodo-Gen was from Pierce, Ltd. (Chester, England). Pepsin was from Sigma (Poole, England). Dow Corning silicone fluids 200/ICS, 200/SCS, and 550 were purchased from Dow Corning Corp. (Midland, MI).

1 The abbreviations used are: dgA, deglycosylated ricin A chain; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; IC50, 50% inhibitory concentration.
Cells. The cell line L540, which derived from a patient with Hodgkin’s disease (20) was maintained in RPMI 1640 supplemented with 20% (v/v) fetal calf serum, 4 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin.

Preparation of dGa. The ricin A chain was purified by the method of Fulton et al. (21). Deglycosylated ricin A was prepared as described previously (22). For conjugation with antibodies or Fab’ fragments, the A chain was reduced with 5 mM DTT and subsequently separated from DTT by gel filtration on a column of Sephadex G-25 in PBS, pH 7.5.

Antibodies. Five monoclonal antibodies which recognize the Ki-1 antigen (CD30) were used in this study: HRS-1, HRS-3, HRS-4, Ber-H2, and Ki-1 (23-25). The characteristics of these antibodies are summarized in Table 1. HRS-1, Ber-H2, and Ki-1 were separated from the ascitic fluid of hybridoma-bearing BALB/c mice by affinity chromatography on staphylococcal protein A-Sepharose. HRS-3 and HRS-4 were purified from ascitic fluid by ammonium sulfate precipitation and ion exchange chromatography on DEAE-Sepharose.

MRC OX7, a mouse IgG1 monoclonal antibody recognizing the Thy-1.1 antigen, was prepared from ascitic fluid as described by Mason and Williams (26) and was used as a nonspecific control antibody. The antibody preparations were >90% pure when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Preparation of Fab’ fragments. HRS-3 and HRS-4 were dialyzed into 0.1 M citrate buffer, pH 8.0, and concentrated by ultrafiltration (Amicon; PM-10 membrane) to 7.5 mg/ml. The pH was reduced to 3.7 by addition of 1 M citric acid and the antibody solutions were subsequently incubated for 4 h at 37°C with pepsin (enzyme:protein ratio, 1:6 by weight). The digestion was terminated by raising the pH to 8.0 with 1 M Tris buffer. The Fab’ fragments were isolated by gel filtration on columns of Sephacryl S-200HR equilibrated in PBS, pH 7.5. F(ab’2)2 fragments were reduced to Fab’ monomers with 1-5 mM DTT. Residual DTT was removed by gel filtration on Sephadex G-25.

Preparation of Immunotoxins. IgG immunotoxin was prepared using the 4-succinimidylisocarbonyl-α-methyl(2-pyridyldithio)toluene linking agent described by Thorpe et al. (27). Briefly, 4-succinimidylisocarbonyl-α-methyl(2-pyridyldithio)toluene dissolved in dimethylformamide was added to the antibody solution (7.5 mg/ml in borate buffer, pH 9.0) to give a final concentration of 0.11 mM. After 1 h the derivatized protein was separated from unreacted material by gel chromatography on a Blue Sepharose CL-6B column and aggregates. Finally, the immunotoxin was separated from free antibody by chromatography on a Blue Sepharose CL-6B column equilibrated in 0.1 M phosphate buffer, pH 7.5, according to the method of Knowles and Thorpe (28).

Fab’ immunotoxins were prepared according to the method of Ghetie et al. (16). Briefly, Fab’ fragments (5 mg/ml in 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA) were derivatized with 5.5’-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent) at a final concentration of 2 mM. Unreacted Ellman’s reagent was removed by gel filtration on a Sephadex G-25 column equilibrated in PBS. The derivatized Fab’ fragments which contained 1-2 activated disulfide groups were allowed to react with a 1.5-fold molar excess of freshly reduced A chain for 2 h at room temperature. The Fab’ dGa immunotoxins were subsequently purified on Sephacryl S-200HR and Blue Sepharose columns as described for IgG immunotoxins.

The A chain component of all the immunotoxins fully retained its ability to inhibit protein synthesis in rabbit reticulocyte lysates (29) after the A chain had been released from the immunotoxins by reduction with DTT.

Radioiodination. Monoclonal antibodies were labeled with carrier-free 125I using the Iodo-Gen reagent to a specific activity of approximately 1 μCi/μg as described (30). Briefly, 500-750 μg of antibody in 100 μl of borate buffer were incubated with 0.7 mCi of Na125I in glass tubes coated with 8 μg of Iodo-Gen for 10 min at room temperature. Free iodide was removed by gel chromatography on a Pharmacia PD-10 column. The radioiodinated antibodies fully retained their capacity to bind to L540 cells, as shown by their ability to compete equally with unlabeled antibodies for binding to cell antigens when applied at saturating concentrations (26).

Cross-Blocking Experiments. Triplicate samples of L540 cells (5 × 10⁶ cells/ml, 100 μl) in PBS containing 2 mg/ml BSA and 0.2% (w/v) NaN₃ (PBS/BSA/N₃) were mixed with 125I-labeled antibody (5 μg/ml, 100 μl) and a 100-fold excess of various unlabeled antibodies. The samples were then incubated for 30 min at 4°C. The cells were washed three times with PBS and the radioactivity of the pellet was measured using a Packard Multi-Prias 1 gamma counter. Blocking of labeled antibody was calculated as

\[
\text{% blocking} = \left(1 - \frac{\text{cpm in presence of blocking antibody}}{\text{cpm in presence of nonspecific antibody (OX7)}}\right) \times 100
\]

Scatchard Analysis. 125I-labeled antibody (50 μl) at various concentrations (0.25-32 μg/ml) was mixed for 1 h at 4°C with L540 cells (2 × 10⁶ cells/ml, 50 μl) in PBS/BSA/N₃. The cells were separated from the supernatant by centrifugation (12,000 × g, 1 min) through 75 μl of a mixture of 8.8% (v/v) Dow Corning silicone fluid 200/1CS, 7.2% 200/5CS, and 84% Dow Corning 550. The Eppendorf tubes were then snap-frozen and the tips containing the cell pellets were cut off. The radioactivity in the cell pellet and in the supernatant were measured.

The amount of radiolabeled nonspecific OX7 antibody that bound to the cells under identical conditions was subtracted from the total amount of radiolabeled specific antibody that was associated with the cells to obtain the amount of specific antibody that was attached to cell antigens. The dissociation constant (Kd) and the number of molecules of antibody bound per cell under equilibrium conditions were calculated by analyzing the data according to the method of Scatchard (31).

Cytotoxicity Assays. L540 cells suspended at 4 × 10⁶ cells/ml in complete medium were distributed in 100-μl volumes into the wells of 96-well flat-bottomed microtiter plates. Immunotoxins in the same medium were added (100 μl/well) and the plates were incubated for 24 h at 37°C in an atmosphere of 5% CO₂ in humidified air. After 24 h, the cells were pulsed with 1 μCi [³H]leucine for another 24 h. The cells were then harvested onto glass fiber filters using a Titerhek cell harvester and the radioactivity on the filters was measured using a liquid scintillation spectrometer (LKB; Rackbeta). The percentage of reduction in [³H]leucine incorporation, as compared with untreated control cultures, was used as the assessment of killing (16).

Immunoperoxidase Staining of Human Tissues. Cryostat sections of normal human tissues were treated with antibodies and stained using the avidin-biotin-peroxidase complex immunoperoxidase method. The three layer immunoperoxidase method, or the APAAP technique that have been described in detail elsewhere (23, 25, 32, 35).

RESULTS

Cross-Blocking of CD30 Monoclonal Antibodies. These experiments were performed to determine whether the five antibodies recognize the same or different epitopes on the CD30 antigen. The results are shown in Fig. 1. HRS-1, HRS-3, HRS-4, and Ber-H2 cross-blocked each others’ binding to L540 cells but were not blocked by Ki-1. HRS-1 was slightly less effective at blocking HRS-3, HRS-4, and Ber-H2 binding than were HRS-3, HRS-4, and Ber-H2 at blocking each others’ binding, probably because it has lower affinity. Ki-1 was blocked only by itself and did not block the other four antibodies.

Thus, there appear to exist at least two epitopes on the CD30

<table>
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<th>Subclass</th>
<th>Immunogen</th>
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<td>L428</td>
<td>Pfundshuh et al. (23)</td>
</tr>
<tr>
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<td>L540</td>
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<td>Ki-1</td>
<td>IgG3</td>
<td>L428</td>
<td>Schwaab et al. (25)</td>
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</table>
antigen, one of which is recognized by HRS-1, HRS-3, HRS-4, and Ber-H2 and the other by Ki-1.

Scatchard Analyses of the Binding of Intact Antibodies and Fab’ Fragments. Table 2 summarizes the results of the Scatchard analyses of the binding of the five intact antibodies and the two Fab’ fragments tested. HRS-4 had the highest avidity for L540 cells (K_a 7 nM). Ber-H2 and HRS-3 had the next highest avidity with K_a values of 14 and 15 nM, respectively. The most weakly binding antibodies were HRS-1 and Ki-1 which had K_a values of 160 and 380 nM, respectively.

As expected from the results of others (16, 17), the intact antibodies bound to L540 cells more avidly than their Fab’ fragments. The difference was 1.8-fold for HRS-3 and 2.4-fold for HRS-4. This is due to the fact that intact antibodies can bind two antigens per cell, whereas the monovalent Fab’ fragments bind to one antigen. In accordance with this, the number of Fab’ molecules bound per L540 cell at saturation exceeds the number of intact antibodies by a factor of 1.6–2.0. The high absolute number of molecules bound (1.6–1.7 x 10^6 for intact antibodies) may be because L540 cells are large, having approximately 8 times the volume of, e.g., human B-lymphocytes.

Cytotoxicity of Immunotoxins to L540 Cells. A representative cytotoxicity experiment is shown in Fig. 2 and the results of several experiments are summarized in Table 3. The IgG immunotoxins fall into two groups. The immunotoxins derived from the high affinity antibodies, HRS-3, HRS-4, and Ber-H2, were powerfully toxic and inhibited protein synthesis by L540 cells by 50% at concentrations (IC_50) of 0.9, 1.0, and 2.0 x 10^-10 M, respectively; by contrast, the immunotoxins derived from the low affinity antibodies, HRS-1 and Ki-1, were weakly effective with IC_50 values of 0.8–1.0 x 10^-8 M, respectively. The potency of the two most powerful immunotoxins, HRS-3.dgA and HRS-4.dgA, was only 15-fold less than that of ricin itself.

The cytotoxic effect of all the immunotoxins was specific since the native antibodies and OX7.dgA, an immunotoxin that does not bind to L540 cells, were not toxic at 10^-6 M.

The immunotoxins prepared from the Fab’ fragments of HRS-3 and HRS-4 were also highly toxic to L540 cells, with IC_50 values of 7.0 and 3.0 x 10^-10 M, respectively. These immunotoxins were therefore only 7.8- and 3.0-fold less toxic, respectively, than their intact IgG.dgA counterparts. The lower activity of the Fab’ immunotoxins is consistent with the findings of others (16, 33, 34) and is explained by the 1.8–2.4-fold lower affinity of the monovalent Fab’ fragments compared with their divalent IgG counterparts.

Immunohistological Staining Pattern of Normal and Malignant Human Tissue. As shown in Table 4, the pattern of reactivity of the five CD30 antibodies was very similar with the exception of HRS-4 which unexpectedly stained normal pancreatic tissue. They all strongly stained Hodgkin’s disease tissue although there was a tendency in the lymphocyte-dominant subtype to give weaker staining (data not shown).

All the antibodies reacted with a few rare cells in the bone marrow, liver, lymph nodes, skin, spleen, and thymus. These cells appeared to be large mononuclear cells in accordance with the finding that the antibodies stain activated lymphocytes (35). No staining was seen in the colon, kidney, or lung.

The strong staining of pancreatic tissue by HRS-4 probably precludes the use of this antibody for therapy. Evidently, antibodies that recognize the same epitope on the immunizing antigen differ in primary sequence in a way that can lead to spurious cross-reactivity with normal tissues. Similar unpredictable cross-reactivity has been observed with CD22 antibodies by Li et al. (36).

DISCUSSION

The major finding to emerge from this study is that CD30 immunotoxins directed against the Ki-1 antigen on Hodgkin
cells have high potency, and specificity of cytotoxic effect in vitro and sufficiently restricted binding to normal human tissues suggest their use for the treatment of Hodgkin's disease in humans.

The Ki-1 antigen was first described by Schwab et al. (25). It is composed of two nonreducible subunits with molecular weights of 105,000 and 120,000 (37). The antibody, which was raised against the Hodgkin cell line L428 (20), was originally thought to be specific for Hodgkin and Reed-Sternberg cells. It has since been demonstrated to be present on anaplastic large cell lymphomas (35), peripheral T-cell lymphomas (35), cutaneous lymphoid infiltrates (38), and tumor cells of embryonal carcinoma (39). The Ki-1 antigen is not expressed on resting mature or precursor B- or T-cells, but it can be induced on these cells by phytohemagglutinin, human T-cell leukemia virus, or Epstein-Barr virus. Since the induction is accompanied by the expression of other activation markers such as HLA-DR, transferrin receptor, and interleukin 2 receptor, it was concluded that Ki-1 identifies both activated normal T- and B-lymphocytes and lymphomas derived from such cells (35). Because the Ki-1 antigen is expressed on all cases of Hodgkin's disease apart from the lymphocyte-predominant subtype (19) and has very limited expression on normal tissue (35), it appears to be a good target for immunotherapy. Only one of the five CD30 antibodies tested in this study (HRS-4) exhibited a strong cross-reactivity with a vital organ (pancreas) that would preclude its clinical use as an immunotoxin.

Cross-blocking studies performed on the five CD30 antibodies used in the present study indicate that there are at least two epitopes on the Ki-1 antigen. One epitope is recognized by HRS-1, HRS-3, HRS-4, and Ber-H2 and the other is recognized by Ki-1. This accords with the findings of Schwarting et al. (24) who demonstrated by fluorescence-activated cell sorter analyses that Ki-1 and Ber-H2 recognize different epitopes and of Pfreibuehlschuh et al. (23) who found no cross-blocking between HRS-1 and biotinylated Ki-1.

HRS-3, HRS-4, and Ber-H2, which bound most strongly to L540 Hodgkin cells (Kd 15, 7, and 14 nM, respectively) formed the most potent IgG.dgA immunotoxins. All three immunotoxins killed 50% of L540 cells at 0.9–2.0 × 10^{-10} M which is only 15–30-fold greater than is needed for an equivalent effect with ricin itself. HRS-1 which binds to the same epitope 11–23-fold more weakly was 40–90 times less active as an immunotoxin. Ki-1, which recognizes a different epitope and has a low affinity comparable to that of HRS-1, also yielded a relatively ineffective immunotoxin. All the immunotoxins retained about 50% of their binding capacity as judged by fluorescence-activated cell sorter analyses (data not shown). Thus, it can be deduced that the affinity (avidity) of these CD30 antibodies rather than the epitope they recognize is the primary determinant of their potency as ricin A-containing immunotoxins. Different conclusions about the importance of epitope location have been drawn from other studies. Shen et al. (17) concluded that both antibody affinity and epitope location determined the potency of CD22 immunotoxins. By contrast, Press et al. (40) in a study of three CD2 immunotoxins concluded that epitope location critically influenced immunotoxin potency; immunotoxins recognizing one epitope on the CD2 molecule were rapidly transported to lysosomes and degraded, whereas an immunotoxin recognizing another epitope lying closer to the membrane remained in peripheral endocytic compartments and was powerfully toxic.

The Fab' fragments of HRS-3 and HRS-4 yielded immunotoxins that were only 7.8- and 3-fold less potent, respectively, than their IgG.dgA counterparts. Their lower activity can be explained by the fact that they can bind to only a single antigen on the cell surface and thus bound 1.8–2.4-fold more weakly than their divalent counterparts. Fab' immunotoxins and IgG immunotoxins have different advantages that recommend their use for therapy. The stronger affinity, greater cytotoxic activity, and longer half-life in vivo are the major advantages of IgG immunotoxins over Fab' immunotoxins (17). The Fab' immunotoxins, on the other hand, may penetrate better into solid tumors (15) and have lower immunogenicity in humans because they lack the relatively immunogenic Fc portion of the antibody.

<table>
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<tr>
<th>Material</th>
<th>Ig_{50} (m)</th>
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Table 4 Reactivity of CD30 antibodies with normal and malignant cells of various tissues

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<tr>
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<td>Liver</td>
<td>±(b)</td>
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<td>n.d.</td>
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* - no staining; +, weak staining; ++, moderate staining; ++++, strong staining; ±, mixed staining pattern as follows: (a) very few positive cells; (b) few positive Kupffer-cell like cells; (c) few positive large cells around, between, and at the inner rim of the follicular mantles; (d) few positive histiocyte-like cells; (e) few positive large cells in the white pulp.

n.d., not determined.
RICH A CHAIN-CONTAINING IMMUNOTOXINS AGAINST CD30 ANTIGEN

(41) We are currently comparing IgG and Fab' immunotoxins for antitumor effects on solid L540 tumors growing in nude mice. These results will be reported at a later date.

In conclusion, ricin A chain immunotoxins constructed from the high affinity CD30 antibodies HRS-3, HRS-4, and Ber-H2 and their Fab' fragments are powerfully and specifically toxic to Hodgkin cells in vitro. Since HRS-3.dGa combines highest potency in vitro and least cross-reactivity with normal human tissues it appears to be the immunotoxin of choice for the treatment of Hodgkin's disease in humans.

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REFERENCES


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Andreas Engert, Francis Burrows, Wolfram Jung, et al.


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