Abstract. Background: The BCL2 proto-oncogene in non-Hodgkin’s lymphoma is a dominant inhibitor of apoptosis. The goal of this work was to develop a 177Lu-labeled anti-BCL2-peptide nucleic acid (PNA) conjugate designed for dual modality NHL therapy, i.e., simultaneous down-regulation of BCL2-mediated resistance to apoptosis and delivery of cytotoxic internally emitted radiation. Materials and Methods: The effect of 1,4,7,10-tetraazacyclododecane-N,N',N''',N'''-tetra-acetic acid (DOTA)-anti-BCL2-Tyr3-octreotate was evaluated by uptake, efflux, proliferation, and viability assays, using Mec-1 lymphoma cells. In vitro dosimetry was modeled with a Monte Carlo projection. Results: Cellular efflux indicated moderate retention of radioactivity in the Mec-1 cells. Viability studies using the 177Lu-labeled PNA conjugate indicated a mass-dose dependence and strongly additive statistical effect in reducing cellular viability. Conclusion: These studies demonstrate the ability of a BCL2 antisense PNA conjugate to specifically target, be retained in, and reduce cellular viability in Mec-1 cells. Viability studies using the 177Lu-labeled PNA conjugate indicated a mass-dose dependence and strongly additive statistical effect in reducing cellular viability. Conclusion: These studies demonstrate the ability of a BCL2 antisense PNA conjugate to specifically target, be retained in, and reduce cellular viability in Mec-1 cells. The results also hold promise for the development of a therapeutic radiopharmaceutical with potential dual modality function.

The protein product of the B-cell lymphoma/leukemia-2 (BCL2) proto-oncogene is a dominant inhibitor of apoptosis. In aggressive non-Hodgkin’s lymphoma (NHL), large cohort studies have shown that overexpression of the BCL2 gene correlates strongly with resistance to radiation and chemotherapy, increased survival of cancer cells, high relapse rate, and poor disease-free and overall survival (1-5). Thus, patients whose tumors are found to overexpress BCL2 might respond better to alternative treatments such as targeted immunotherapy, radioimmunotherapy, or antisense therapy, all of which act through mechanisms that down-regulate BCL2. Human NHL also expresses type 2 somatostatin receptors (SSTR2) in approximately 87% of cases (6). Previous work indicated that 177Lu-1,4,7,10-tetraazacyclododecane-N,N’,N”-tetra-acetic acid (DOTA)-Tyr3-octreotate, a somatostatin-like radiopharmaceutical, provides effective, selectively targeted radiotherapy in human SSTR-expressing tumors (7-12). Furthermore, this peptide is an attractive vehicle for delivery of intracellular tumor-targeting agents, such as those designed to act against BCL2 (13-16).

Lutetium-177 is a very promising therapeutic radionuclide. A readily available reactor-produced lanthanide, it has several very favorable physical properties suitable for targeted radiotherapy. With a 6.71 day half-life, the principal mode of decay is β– emission with a maximum energy of 497 keV (90%) and two low abundance γ emissions of approximately 113 (6%) and 208 (11%) keV respectively, suitable for tracking radiopharmaceuticals and for radiation dosimetry in vivo (17, 18). Additionally the coordination chemistry of the radiometal lends itself to forming stable complexes with DOTA quite readily (18).

The present work addresses the hypothesis that combined 177Lu and BCL2 antisense therapy act synergistically or additively with respect to cell proliferation and viability in an in vitro model of B-cell lymphoma. In vitro uptake, efflux, proliferation, and viability assays were designed to assess the targeting of a 177Lu-labeled BCL2 antisense peptide nucleic acid (PNA)–peptide conjugate (Figure 1), against human NHL cells in suspension culture. Previous work described an 111In-labeled BCL2 antisense PNA–peptide conjugate, using Tyr3-octreotate for SSTR2 receptor-mediated delivery for the molecular imaging of BCL2 in cell and mouse models of NHL (19). The results of those experiments demonstrated specific tumor targeting, imaging, and reduction of BCL2 protein synthesis by 51% in...
acetic acid (DTPA), pH 6.0, was then added to a final concentration
continuous mixing. An aliquot of 10 mM diethylenetriamine
tetraacetic acid (TFA) (87.5%) and 2.5% of each of
were deprotected and detached from the resin by treatment for 4 h
end of the synthesis using DOTA tris-
Gallazzi
specifically targeting
mRNA for targeting, served as the tumor cell model for the
present studies. This article reports the development of a
cytotoxic $^{177}$Lu-labeled antisense agent capable of
Material and Methods
PNA–peptide synthesis. DOTA-Tyr$^3$-octreotate was synthesized on
an Advanced ChemTech (Louisville, KY, USA) 396-V multiple
synthesizer, using standard Fmoc chemistry. Peptidyl-resins were
transferred to a manual reaction vessel where PNA synthesis was
performed using modifications of methods described previously by
Gallazzi et al. and Lewis et al. (20, 21). DOTA was coupled at the
end of the synthesis using DOTA tris-tert-butyl ester. The conjugates
were deprotected and detached from the resin by treatment for 4 h
with trifluoroacetic acid (TFA) (87.5%) and 2.5% of each of the
following scavengers: m-cresol, H$_2$O, phenol, thioanisole, 1,2-
ethanediol, and trisopropylsilane. Precipitation and multiple
washing with diethyl ether yielded the crude conjugates, which were
analyzed by liquid chromatography–electrospray ionization mass
spectrometry (LC-ESI-MS). Disulfide cyclization was accomplished
by dimethyl sulfoxide (DMSO) oxidation of crude conjugates. Each
compound was purified by semi-preparative reversed phase HPLC
(RP-HPLC), using a stepwise gradient of 0-20% solvent B (solvent
A: 0.1% TFA/H$_2$O; solvent B: 0.1% TFA/CH$_3$CN) in 30 min.
Fractions corresponding to the desired products were pooled, lyophilized and analyzed for
purity by LC-ESI-MS. Typical purity of the PNA–peptide conjugate
was found to be between 95 and 98%.
$^{177}$Lu-labeling of conjugates. The representative conditions for
labeling the DOTA–PNA–peptide conjugate with $^{177}$Lu given here
were based on previously published techniques (19, 21). To 185 MBq
of $^{177}$LuCl$_3$ in 150 μl of 30 mM sodium acetate/25 mM sodium
ascorbate, pH 5.0, was incubated with 1 μg of DOTA-Tyr$^3$-
octreotate at 99˚C for 30 min. Radiometal incorporation and
radiochemical purity (typically 98%) were determined by radio-thin-
layer chromatography (radio-TLC) (22). The radiolabeled peptide
was diluted with normal saline for addition to the cellular
suspending solution with further purification.

**Cell culture.** The human non-Hodgkin’s lymphoma cell line Mec-1
(DSMZ Cell Lines Bank, Braunschweig, Germany) was cultured in
a modified RPMI-1640 medium containing 1 mM sodium pyruvate,
10 mM HEPES, 2 mM L-glutamine, 0.4 mg/ml gentamicin and
supplemented with 10% heat inactivated fetal bovine serum at 37˚C
in a 5% CO$_2$ atmosphere. Cell passage numbers were deliberately
kept below 10, as unpredictable growth and large-scale colony death
were observed in flasks containing cells with passage numbers
greater than 15. Passage numbers fewer than 10 were consistently
monitored to maintain cellular viability and predictable confluent
growth periods. All cell culture methodologies were based on the
work of Jia et al. (19).

**Cell uptake and efflux assays.** The methods for cell uptake and
efflux were based on the previously published techniques of Jia et al.
and Lewis et al. (19, 21). For uptake studies, aliquots of 37 kBq
of the $^{177}$Lu-labeled conjugates were added to $1 \times 10^7$ Mec-1 cells in
5 ml of serum-free media. During incubation at 37˚C and 5% CO$_2$,
triplet aliquots were removed at various time points from 1 min
to 4 h. Cells were isolated by centrifugation, washed, and counted
separately from the liquid phase to determine the percentage uptake
of the radiopharmaceutical. The cell-associated radioactivity in each
sample phase (pellet, supernatant, and wash) was counted on a
gamma counter. Uptake was then calculated by dividing the pellet-
associated radioactivity by the total radioactivity. Cell viability after
4 h of incubation was determined to be ~98% by Trypan Blue (Thermo Fisher Scientific, Waltham, MA, USA) exclusion and hemacytometry.

For efflux studies, radiopharmaceutical uptake was performed as described above for 2 h at 37°C and 5% CO₂. Cells were then isolated by centrifugation, washed with fresh medium to remove residual radioactivity, and re-suspended in fresh medium. Efflux of 177Lu from the cells was measured at subsequent time points from 1 min to 4 h, after isolating aliquots of the cell pellets and counting the radiation in them separately from the supernatants and washes, as described above.

**Radiation dosimetry.** The radiation-absorbed dose delivered by the 177Lu-labeled PNA–peptide conjugate was calculated using a Monte Carlo ‘n’ particle model (MCNP5) (23). This Fortran-based Monte Carlo projection uses multiple iterations of random modeled decay events to estimate the dose to the nucleus or cytoplasm based on the location of the decay and the geometry of the total closed system. The assumptions for this model were that the Mec-1 cells have an average radius of 4 μm, a nuclear volume of approximately 40%, and a cell volume fraction of 0.15% of the total water matrix. An MCNP model was calculated using the β⁻ emission of 177Lu. Due to the planar arrangement of the cells in the wells of plates, the γ emission of 177Lu was not taken into account. The dosimetry calculations were used to normalize the absorbed doses of the radiopharmaceuticals (24, 25).

**Proliferation and viability assays.** Cells were counted daily using the Alamar Blue (Invitrogen, Carlsbad, CA, USA) assay on a fluorescence spectrometer operated at 530 nm/590 nm. Cells were also counted daily on a hemacytometer. The non-viable fraction was recorded after the cells were diluted with 0.4% Trypan Blue vital stain as per the manufacturer’s instructions. Dilutions were appropriate to yield a minimum 100 cell count. Three replicates allowed detection of a 25% difference with a statistical power of 0.80 and an α value of 0.05. Results from the Alamar Blue assay were validated to within 2% of those of conventional hemacytometry.

During the viability and proliferation assays, the amount of each compound added was varied as a function of mass and the radiation dose administered. Doses of 0, 0.25, 0.50, 1.0, and 2.0 Gy and from 0, 2, 5, and 10 μg of compound, respectively, were evaluated. Comprehensive studies (n=3) were conducted for each experimental category. Each well in a sterile 24-well plate was inoculated with 4×10⁵ Mec-1 cells, incubated in 1 ml of medium, and treated according to its respective experimental category. Cellular response was monitored using Alamar Blue, as well as Trypan Blue exclusion and hemacytometry.

**Statistical analysis.** Utilizing a two-way analysis of variance (ANOVA) without replication in conjunction with a randomized complete block design for the study allowed for the collection and analysis of sufficient data by treating each well as a distinct block in the treatment instead of as a block replicate. Furthermore, since the samples were seeded from the same original source, it was possible to pool the data and manipulate by treatment type and day.

The use of a post-hoc comparison of effects by a Bonferroni test of significant difference allowed for the resolution of effects both within and between treatments or study conditions.

**Results**

Cellular uptake and efflux assays were used to determine the amount of the PNA compound that was residualized intracellularly. The results of these assays are shown in Figure 3 A and B. The uptake results displayed the anticipated second-order shaped curve indicative of a cellular saturation process, with maximal uptake values reaching 2.5% of the total radioactivity at 4 h. The efflux data showed that by the 4-h time point, 30% of the compound was still retained in the tumor cells. An integration of uptake and

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Figure 2. RP-HPLC chromatograms of the crude (top) and peak purified (bottom) 177Lu-labeled PNA compound.
Efflux data over time allowed dosimetry calculations to be made utilizing an MCNP5 projection. Incorporating the values calculated by the program into the following equation:

\[ \text{Decay} = \frac{\text{Act}_0 (\text{Bq})}{\lambda (\text{s}^{-1})} (1 - e^{-\lambda t}) \]

where \( \text{Act}_0 \) (in Bq) is the initial radioactivity in the volume of interest, \( \lambda \) (in s\(^{-1}\)) is the decay constant (=ln2/T\(_{1/2}\)), and \( t \) is the elapsed time from initiation of the study. This equation allowed for calculation of self and cross doses from nucleus, cytoplasm, and water matrix, respectively (Table I). This analysis also allowed for the calculation of the number of kBq of \(^{177}\)Lu required to prepare test conditions of 0.25, 0.5, 1.0, and 2.0 Gy (Table I). Additionally, masses of 2, 5, and 10 μg of the PNA–peptide were tested for optimal effect. Choice of PNA masses was made based on internalization potential relative to the number of somatostatin cell surface receptors. Mass- and dose-variable proliferation and viability studies were designed to evaluate the optimal combination of radiation-absorbed dose and mass of antisense PNA compound, in an effort to affect tumor cell death via radiologic and biologic means, respectively.

Assays designed to assess the proliferative effects of varied PNA mass and \(^{177}\)Lu absorbed dose of the labeled PNA–peptide conjugate yielded no statistically significant results (Figure 4). However, viability assays of varied PNA mass and \(^{177}\)Lu absorbed dose of the labeled PNA–peptide conjugate indicated mass- and dose-dependent responses. The effects on cellular viability were graphically observed in the stratification of the data, with the mass of PNA administered correlating to the observed level of decrease. This effect was most noticeable in the 2 Gy/10 μg test condition, although also observed to a lesser extent at the lower absorbed dose/PNA mass test conditions of \(^{177}\)Lu-PNA-peptide (Figure 5). At 2 Gy/10 μg, the percentage of viable cells decreased by 35.8% from day 0 to day 3, as compared to the 24.2% reduction in viability over the same time period with 10 μg of unlabeled PNA–peptide. Statistical
analysis of the observed reduction in viability, relative to mass and dose administered, indicated a significant difference when compared to the unlabeled PNA–peptide control (day 1 \( p < 0.003 \), day 2 \( p < 0.005 \), day 3 \( p < 0.002 \)).

Additional results also suggest an additive relationship between the observed efficacy of the mass of PNA and the dose of radiological insult administered, as compared to the combination of unlabeled PNA–peptide and \( ^{177}\)Lu-DOTA-Tyr\(^3\)-octreotate (Figure 6). When treated with 2 Gy/10 μg of the \( ^{177}\)Lu-labeled PNA–peptide, the percentage of viable cells decreased by 35.8% from day 0 to day 3, as compared to the 28.6% reduction in viability appreciated in the combination of unlabeled PNA–peptide and \( ^{177}\)Lu-Tyr\(^3\)-octreotate. Statistical analysis indicated a significant difference (day 1 \( p < 0.005 \), day 2 \( p < 0.004 \), day 3 \( p < 0.002 \)) for the 2 Gy/10 μg test condition, providing confirmation of the additive relationship.

**Discussion**

Leveraging the anti-apoptotic functionality of the \( BCL2 \) proto-oncogene along with the overexpression of SSTR2 in human NHL allowed us to demonstrate the feasibility of a novel site-directed therapeutic agent. A biological effect was achieved using somatostatin peptide analogues as delivery vehicles for an anti-\( BCL2 \) agent in receptor mediated uptake, resulting in a specific antisense effect inhibiting the translation of \( BCL2 \) mRNA and thus down-regulating the anti-apoptotic function of the protein (19). In the present work, a radiological insult was achieved by labeling the PNA–peptide conjugate with cytotoxic lutetium-177, with a primary \( \beta^- \) emission of 497 keV.

Uptake and efflux assays demonstrated that the compound was retained in Mec-1 cells after 4 h of elapsed time. Administration of the PNA–peptide conjugate at 2 Gy and in varying masses showed a direct correlation between cell death and the mass of PNA–peptide administered, with the most significant effect being observed with the largest tested mass, 10 μg. Likewise, a direct correlation was observed between cell death and the radiation-absorbed dose of \( ^{177}\)Lu administered.

Proliferation assays did not show a generalized decrease in cellular proliferation over the course of the study in each test condition. Thus, the statistically insignificant results generated may have been the result of a different causal relationship. We believe that the different effects on proliferation and viability were actually due to different cellular responses. Cell death following internal emitter therapy often results primarily in apoptotic, as opposed to necrotic, cell death. It is expected that apoptosis would occur over the course of several days under the conditions employed, affecting the observed viability of the colony as opposed to its proliferation over a three-day period.
period. The observation of any proliferative effects resulting from necrotic cell death would have necessitated a dramatic increase in the length of time that the study was allowed to run. This was counterproductive to the determination of reproducible results, as the physical dimensions or maximum volume of each well dictates the length of time that the seeded cells remain viable.

The observation that an increase in the mass of PNA administered translated to an increased antisense effect or mass/dose response was most readily associated with the 10 μg/2 Gy test condition. These results led to the observation that combined effects of 177Lu radiotherapy with BCL2 antisense therapy acted with strongly additive characteristics in reducing viability, as compared to the combination of unlabeled PNA–peptide and 177Lu-Tyr3-octreotate.

One plausible hypothesis as to the source of the observed additive effect can be derived from the characteristics of the 177Lu radionuclide as it decays to 177Hf. As previously discussed, the predominant decay emission of 177Lu is a β−-particle; however, there are two associated γ emissions with respective energies of 113 keV and 208 keV. Newly reported data suggest the emission of an average of two internal conversion (IC) electrons as a commonly utilized alternative branch to the 113 keV γ emission (26). We have demonstrated a measurable reduction in cellular viability with the administration of the 177Lu-Tyr3-octreotate, which is only capable of targeting SSTR2 surface receptors. We attribute the reduction in cellular viability caused by this agent to DNA damage caused by the β−-particle emission. Furthermore, our data have demonstrated that the unlabeled PNA–peptide has some effect on viability reduction which is due to the direct biological action of the compound. One hypothesis generated to explain the observed additive effect is that combined effects of 177Lu radiotherapy with an antisense effect. Work is currently underway to evaluate the 177Lu-labeled anti-BCL2–PNA–peptide agent in a tumor-bearing mouse model.

The current series of experiments has displayed definite promise for the development of a corresponding multimodality agent for targeted radiotherapy with a combined antisense effect. Work is currently underway to evaluate the 177Lu-labeled anti-BCL2–PNA–peptide agent in a tumor-bearing mouse model.

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References


Table I. Table of dosimetric calculations based on values obtained from the MCNP5 projection analysis of uptake and efflux data.

| Location of Dose to nucleus Dose to cytoplasm Relative dose Activity Activity | Location of Dose to nucleus Dose to cytoplasm Relative dose Activity Activity |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Lu-177 (Gy/decay)       | (Gy/decay)               | (Gy)                     | (kBq)                   | (μCi)                   |
| Nucleus                  | 0.0021                   | 0.0008                   | 0.25                    | 1.57×10⁻²               | 4.25×10⁻⁴               |
| Cytoplasm                | 0.0008                   | 0.0012                   | 0.5                     | 3.14×10⁻²               | 8.50×10⁻⁴               |
| Water matrix outside of the cell | 0.0011                 | 0.0013                   | 1                       | 6.29×10⁻²               | 1.70×10⁻³               |
| Total dose per decay (Gy) | 0.004                   | 0.0033                   | 2                       | 1.26×10⁻¹               | 3.40×10⁻³               |


