Non-replicating rhabdovirus-derived particles (NRRPs) eradicate acute leukemia by direct cytolysis and induction of antitumor immunity

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Rhabdoviruses (RVs) are currently being pursued as anticancer therapeutics for various tumor types, notably leukemia. However, modest virion production and limited spread between noncontiguous circulating leukemic cells requires high-dose administration of RVs, which exceeds the maximum tolerable dose of the live virus. Furthermore, in severely immunosuppressed leukemic patients, the potential for uncontrolled live virus spread may compromise the safety of a live virus approach. We hypothesized that the barriers to oncolytic virotherapy in liquid tumors may be overcome by administration of high-dose non-replicating RVs. We have developed a method to produce unique high-titer bioactive yet non-replicating rhabdovirus-derived particles (NRRPs). This novel biopharmaceutical is multimodal possessing direct cytolytic and immunomodulatory activity against acute leukemia. We demonstrate that NRRP resistance in normal cells is mediated by intact antiviral defences including interferon (IFN). This data was substantiated using murine models of blast crisis. The translational promise of NRRPs was demonstrated in clinical samples obtained from patients with high-burden multidrug-resistant acute myeloid leukemia. This is the first successful attempt to eradicate disseminated cancer using a non-replicating virus-derived agent, representing a paradigm shift in our understanding of oncolytic virus-based therapies and their application toward the treatment of acute leukemia.

**Keywords:** acute leukemia; oncolytic virus; non-replicative rhabdovirus-derived particles; NRRP

**INTRODUCTION**

Rhabdoviruses (RVs), such as vesicular stomatitis virus (VSV) and Maraba, are currently being explored as anticancer therapeutics.1–4 Through cell lysis and activation of antitumor immune responses,5,6 live RVs are multitasking self-amplifying cytolytic agents. In tumors, viral propagation is enabled by disrupted metabolic activities7,8 and impaired antiviral programs.9,10 Typically, oncolytic viruses are genetically altered to reduce virus replication in healthy tissue, constraining viral spread to the tumor microenvironment. Although live RVs are being pursued to treat a wide variety of tumor types, their application in hematopoietic malignancies is complicated by several factors. Limited virion production and reduced spread between leukemic cells requires high-dose viral therapy to overcome these inefficiencies. However, uncontrolled live virus spread and off-target effects in normal tissue compromise the safety of this approach, particularly in immunosuppressed patients.11

In the RV field, all oncolytic platforms developed to date utilize a replication-competent virus that spreads between tumor cells.1–4 The dogma pillar to these therapies is that virus replication is a prerequisite for treatment efficacy.12–14 Indeed, reports describing the use of live replication/expression-competent RV as a direct virotherapy for cancer, typically compare efficacy with non-expressing virus controls where no measurable efficacy is observed.1–15 In these studies, the control virus is treated with a substantial dose of ultraviolet (UV) irradiation to intentionally generate an inert bioparticle. We posited that if one could devise a method to generate non-replicating particles, which maintain both oncolytic and immunogenic properties, many of the above barriers in the treatment of hematopoietic malignancies may be overcome.

**MATERIALS AND METHODS**

**Cells**

L1210, A20, A301, Jurkat, HL60 cell lines were from American Type Culture Collection (Manassas, VA, USA), and maintained in suspension culture, Dulbecco’s modified Eagle’s medium-high glucose (HyClone, Logan, UT, USA), with 10% fetal calf serum (CanSera, Etobicoke, ON, Canada), at 37°C and 5% CO2. Cells in culture were maintained at a concentration between 0.5 and 1.0 x 106 cells/ml. Vero cells (kidney epithelial cells extracted from an African green monkey), GM38 (normal human fibroblast cell line) and human dermal fibroblast- adherent cells were from American Type Culture Collection and propagated in same culture media used for the suspension cell lines. Normal bone marrow cells acquired from STEMCELL Technologies Inc. (Vancouver, BC, Canada) were obtained from a healthy volunteer and consented to an approved institutional review board protocol. Acquisition of human leukemic patient samples was approved by the institutional review board of the Ottawa Hospital Research Ethics Board. All patients provided written informed consent for the collection of samples and subsequent analysis.

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Virus
The Indiana serotype of VSV and wild-type Maraba used throughout this study were propagated in Vero cells, as previously described.16 VSV titration was performed using serial dilutions in Dulbecco’s modified Eagle’s medium. Hundred microlitres of each dilution was applied to a confluent monolayer of Vero cells for 45 min. Subsequently, the plates were overlaid with 0.5% agarose in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and the plaques were grown for 24 h. Carnoy’s fixative (methanol:acetic acid in a 3:1 ratio) was then applied directly on top of the overlay for 5 min. The overlay was removed and the fixed monolayer was stained with 0.5% crystal violet for 5 min, after which the plaques were counted.

Viability assays
Viability assays were done in a 96-well plate format. Cells were plated at specific density and treated with live VSV virus or non-replicating rhabdovirus-derived particles (NRRPs). After 72 h, cytotoxicity was assessed by adding 25 μl of alamar blue reagent (ABD Serotec, Raleigh, NC, USA).

Modeling
The model used to describe NRRPs specificity against cells with defects in antiviral signaling pathways was adapted from our previous work described in Le Boeuf et al.17 In brief, this model is represented by a subset of six ordinary differential equations describing the transition between the cell populations (UP, IP, AP and PP) depending on the concentration of NRRPs (N) and interferon (IFN) in the environment. These equations are:

\[
\frac{dUP}{dt} = -K_{UP}\times[N]\times[UP] - \left(\frac{-K_{UP,IFN} + K_{UP,off}}{1 + \frac{EC_{50,IFN}}{EC_{50}}}\right)\times[UP] + K_{UP,off}\times[PP],
\]

\[
\frac{dIP}{dt} = K_{IP}\times[N]\times[UP] - \left(\frac{-K_{IP,IFN} + K_{IP,off}}{1 + \frac{EC_{50,IFN}}{EC_{50}}}\right)\times[IP] - \gamma_{C}\times[IP],
\]

\[
\frac{dAP}{dt} = \left(\frac{-K_{AP,IFN} + K_{AP,off}}{1 + \frac{EC_{50,IFN}}{EC_{50}}}\right)\times[IP] - K_{AP}\times[AP] - \gamma_{C}\times[AP],
\]

\[
\frac{dPP}{dt} = \left(\frac{-K_{PP,IFN} + K_{PP,off}}{1 + \frac{EC_{50,IFN}}{EC_{50}}}\right)\times[UP] + K_{PP,off}\times[AP] - K_{PP,off,IFN}\times[PP].
\]

The parameters used in the above equations represent the concentration of each cell type (\(K_{cell}\)), the rate of IFN-signaling activation (\(K_{IFN,off}\)), the rate of IFN-signaling inactivation (\(K_{IFN,off}\)), the EC50 of IFN (EC50), the rate of cell death (\(\gamma_{C}\)) and the rate NRRP clearance (\(K_{N}\)). The next subset of equation describes the dynamics of NRRPs (N) and IFN whereby:

\[
\frac{dN}{dt} = -K_{N}\times[N]\times[UP] - \gamma_{N}\times[N],
\]

\[
\frac{dIFN}{dt} = K_{IFN}\times[IP] + K_{IFN,1}\times[AP] + K_{IFN,2}\times[PP] - \gamma_{N}\times[IFN].
\]

The parameters described in the above equations represent the rate of NRRP internalization (\(K_{off,IFN}\)), NRRP degradation (\(\gamma_{N}\)), IFN production from IP, AP and PP (\(K_{IFN,1}, K_{IFN,2,1}\) and \(K_{IFN,2,2}\) respectively) and IFN degradation (\(\gamma_{IFN}\)).

The Monte Carlo simulation was performed by randomly varying the above parameters within a 1 log window (see Supplementary Table 1) surrounding physiological parameter derived from literature and experimental evidence.17 Simulations were performed in Matlab using ODE15s imposing a non-negativity constraint. Trends described in Figure 3b represent the median value over 2000 simulations. The number of cells used in these simulations was 2.5E5, the media volume was set at 1 ml and the particle to cell ratio was set at 100. In these simulations, defects in IFN-signaling pathways were simulated by decreasing \(K_{IFN,1}, K_{IFN,2,1}, K_{IFN,2,2}, K_{IFN,off,IFN}\) from 100 to 1% of their original value.

Flow cytometry
Anti-CD33-PerCP Cy5.5 and Anti-CD33-PCS antibodies obtained from eBioscience (San Diego, CA, USA) were used. Annexin-V and 7-AAD viability dye were obtained from eBioscience and performed according to manufacturer’s instructions. Flow cytometry was acquired using a CyAn ADP (Beckman Coulter, Brea, CA, USA). Analysis was performed using the Kaluza software version 1.1 (Beckman Coulter).

Cytokine array
Bio-Plex Pro mouse luminescent-based multiplex cytokine assay (Bio-Rad, Hercules, CA, USA) was performed on mouse serum 20 h after NRRP treatment. The assay was performed according to the manufacturer's instructions.

Murine experiments
For the L1210 vaccination strategy, cohorts of DBA/2 mice (Charles River, Wilmington, MA, USA) received three weekly intravenous doses of 1 × 10^6 γ-irradiated L1210 cells pretreated, or not, with NRRPs. One week following this regimen, a L1210 leukemic challenge (1 × 10^6 cells) was administered via tail vein, and survival was followed. Mice were euthanized upon development of predetermined signs of advanced leukemia end points. For the L1210 treatment strategy, DBA/2 mice were challenged with 1 × 10^6 dose of L1210 blast cells. The following day, mice began a regimen of 3 × 10^7 NRRPs administered intravenously for 3 consecutive days, or treated with live VSV at the maximum tolerable dose (MTD) of 2 × 10^6 viruses per injection, and survival was followed. Institutional guidelines and review board for animal care (The Animal Care and Veterinary Service of the University of Ottawa) approved the above animal studies.

RESULTS
Generation of high-titer NRRPs
We theorized that UV photonic damage of RVs could be used to generate a non-replicating particle that retained bioactivity. In the past, high-dose UV irradiation has been used to ablate the RV genome rendering a biologically inert virus.1,15 We hypothesized that when applied at moderated doses, UV irradiation could be used to generate a quasispecies where replication is lost yet biological activity is maintained. To investigate this possibility, samples of purified VSV-expressing green fluorescence protein (GFP) were exposed to a wide range of UV irradiation intensities and the biological effects of this treatment were examined. When irradiated at a low dose (250mJ/cm²), VSV-eGFP lost its expression capabilities, yet maintained potent cytotoxicity against our immortalized production cell line (Vero; Figures 1a and b). Titering for virus following UV treatment confirmed that the resulting particles were unable to replicate in these cells in sharp contrast with live virus infection (Figure 1c). This effect was equally observed when using other members of the RV family including Maraba (Supplementary Figure 1).

Dose–response curves provide a better understanding of the relationship between cytotoxicity and the irradiation procedure. In these experiments, we observed that only a narrow window of UV fluence allows for the NRRP to maintain cytotoxicity against immortalized cells (Figure 1d), and that this effect requires a particle to cell ratio >10 (Figure 1e). By comparing and contrasting with normal neonatal human dermal fibroblasts (Figures 1d and e), it appears that cytotoxicity is tumor specific. Indeed, normal cells appear to require at least a 10 times higher dose to become sensitive to NRRP-mediated cytotoxicity.

NRRPs are an efficient treatment against leukemic cells in vitro
We next examined whether acute leukemic cells were equally associated with an increased sensitivity to NRRPs. In these experiments, we first compared the cytotoxicity induced in the acute lymphoblastic L1210 cell line with that observed in normal cells. Indeed, normal cells appeared to require at least a 10 times higher dose to become sensitive to NRRP-mediated cytotoxicity.
The classic apoptotic phenotype was observed in leukemic cells characterized by a reduced cell diameter, a shriveled appearance with numerous apoptotic bodies and fragmented nuclear content. To validate the cell death mechanism, we quantified the level of apoptosis in L1210 cells by flow cytometry. In these experiments, we observed potent apoptosis in NRRP-treated samples 30 h after treatment, where 84% of the population was in an early- or late-stage apoptosis (Figure 2b).

Cytotoxicity was next quantified using a standard resazurin assay in a panel of human and murine cell lines representative of hematological malignancies and normal tissue. In these experiments, acute leukemias were highly susceptible to NRRP-mediated cell death while preserving the viability of normal cells (Figure 2c, Supplementary Figure 2).

Modeling depicting NRRPs’ antitumor specificity
To investigate the mechanism by which specificity against tumor cells is achieved, we simulated the cytotoxicity induced by NRRPs in normal and tumor cells. Recently, we have developed a population-based model describing the relationship between cytotoxicity and live oncolytic virus replication dynamics in normal and tumor cells. According to this model (Figure 3a), an infection cycle begins as the uninfected population of cells (UP) encounters virions. This allows the population to become infected, where in the context of live virus, virions and the cytokine known as IFN are released into the environment. As IFN gradually increases, the population of cells activates antiviral signaling (AP), which over time allows this population to clear the viral infection and become protected against further insult (PP). To adapt this model to NRRPs, we removed virus replication dynamics from the model, and simulated the relationship between NRRP-mediated cytotoxicity and the extent of defects in IFN-signaling pathways, a process known to occur in ~80% of cancers. These defects were simulated by decreasing the rate of IFN production, the rate of activation of IFN signaling and the rate of NRRP clearance between tumor and normal cells. To ensure that this observation is systematic, a Monte Carlo simulation platform was utilized. Here, all kinetic parameters were varied within a 1 log window surrounding estimates derived from literature or experimental evidence (Supplementary Table 1). Following simulation across 1000 random parameter pairings (Figure 3b), we observed that as the cancer cells lose their ability to signal or respond to IFN, these cells become more sensitive to NRRP-mediated cytotoxicity. To validate this observation, we investigated the impact of IFN on NRRP-mediated cytotoxicity in normal (human dermal fibroblast) and leukemic (L1210) cells. Interestingly, although the intron A (recombinant IFN) could further increase normal cell protection against NRRP insult (Figure 3c), it had no detectable impact on leukemic cells (Figure 3d).

NRRP activity in AML blast crisis
The translational potential of the NRRP platform was investigated in human clinical samples. Peripheral blood mononuclear cells were obtained from two patients with high-burden acute blast crisis. The patients had circulating blasts with a CD33+ phenotype. Both had previously received extensive treatment for chronic myeloid leukemia and developed multidrug resistance. Similar to our observation in L1210 blast cells, patient samples cultured to enrich for the CD33+ fraction developed obvious NRRP-induced apoptosis with classic morphology (Figure 4a). Indeed, the leukemic CD33+ cells within this population avidly bound the apoptotic marker Annexin-V (Figure 4b). Use of the...
noncultured patient samples was used to evaluate the specificity of this response. In both patients, the preponderant leukemic CD33\(^+\) population was ablated following NRRP treatment, leaving normal cells to dominate the sample (Figure 4c). To ensure that NRRPs do not affect normal white blood cells, the bone marrow mononuclear cells isolated from a healthy donor were treated with phosphate-buffered saline (PBS) or NRRPs. At both early (18 h) and late (65 h) time points, NRRPs did not appear to induce apoptosis within these samples (Figures 4d and e).

NRRPs antileukemic activity \textit{in vivo}

A murine model of leukemic blast crisis was used to evaluate the potential of NRRPs as a therapeutic agent. In brief, on day 1, DBA/2 mice were challenged with 1 \times 10^6 dose of L1210 blast cells. The following day, mice began a regimen of 3 \times 10^7 NRRPs administered intravenously for 3 consecutive days, and survival...
was monitored. In parallel, separate cohorts of mice were treated with live VSV at the MTD of $2 \times 10^6$ viruses per injection or PBS under the same treatment schedule. NRRP-treated mice achieved 80% survival up to day 40, representing a significant advantage versus those treated with PBS ($P < 0.0045$) or live virus ($P < 0.044$). Indeed, mice treated with PBS or live virus, all succumbed to overwhelming leukemia (Figure 5a). NRRPs were well tolerated and administered at the maximal feasible dose for this particular experiment, which represented a 1500 times higher dose than the MTD of live virus. Given that acute leukemia frequently disseminates to the central nervous system, and that wild-type VSV is highly neurotoxic, intracranial injections of NRRPs and live virus were performed. Although mice could tolerate the maximum production dose for intracranial injections of $1 \times 10^8$ NRRPs, all mice rapidly succumbed to a $1 \times 10^4$ dose of live virus.

Prompted by the superlative efficacy and differential MTD afforded by the NRRP therapy, we wondered whether the recipient’s immune system is activated following NRRP administration. To answer this, the peripheral blood serum was collected from L1210 tumor-bearing mice 20 h after treatment with PBS or NRRPs (Figure 5b). In this analysis, we observed that multiple cytokines typically known to recruit and differentiate T cells are induced and circulating following NRRP treatment. Examples of such immunomodulatory cytokines significantly induced by NRRP treatment include leukemia inhibitory factor, interleukin-2, interleukin-4, monocyte chemotactic protein-1, regulated on activation normal T cell expressed and secreted (RANTES), and macrophage inflammatory protein-1α (Figure 5b).

To confirm immune system stimulation, in particular T-cell activation, we adopted a vaccine strategy as previously described. Experimentally, this platform consists of injecting apoptotic cells into immunocompetent animals and measuring protective adaptive immunity against subsequent tumor challenge. We adopted this classical experimental approach to explore whether NRRPs trigger immunogenic apoptosis. Two cohorts of DBA/2 mice (syngeneic to L1210) received three
weekly intravenous doses of 1 × 10^6 γ-irradiated L1210 cells pretreated with NRRPs. Another cohort received the same number of γ-irradiated L1210 cells. One week following this regimen, a L1210 leukemic challenge (1 × 10^6 cells) was administered via tail vein, and survival was recorded. The cohort receiving NRRP-treated L1210 cells had 80% protection after the leukemic challenge, which was otherwise uniformly lethal in the untreated L1210-treated cohorts (Figure 5c). Surviving mice were kept for >150 days to ensure long-lasting protection. These results are consistent with the notion that NRRP-treated acute leukemic cells undergo immunogenic apoptosis.

DISCUSSION
This is the first successful attempt to eradicate disseminated cancer using non-replicating virus-derived particles, and represents a paradigm shift in the field of oncolytic virus-based therapeutics. Through in silico and in vitro testing, we demonstrate that NRRPs, analogous to live virus, are tumor selective, given that they exploit defects in innate immune pathways common to most tumors. However, this platform is unencumbered by the principle safety concern associated with live virus replication, that is, the potential for uncontrolled viral spread in immunocompromised patients. Indeed, the superior safety margin afforded by the NRRP platform was exemplified by the observation that high-titer intracranial NRRP administration was well tolerated by murine recipients.

The outcome for the majority of adult patients suffering from acute lymphoblastic or acute myeloid leukemia remains dismal.25,26 For a minority of patients, allogeneic hematopoietic stem cell transplantation (HSCT) after myeloablative conditioning (HSCT) is potentially curative; however, this procedure is associated with frequent adverse events and significant treatment-related mortality.25 For many patients with chronic-phase CML, targeted tyrosine kinase inhibitor therapy offers excellent disease control.27 However, when progression into acute blast crisis occurs, very limited therapeutic options exist owing to development of multidrug resistance and the rapid kinetics of this form of recalcitrant leukemia.28 Clearly, new and innovative therapeutic approaches are urgently required.

Here we establish that NRRPs exhibit both direct cytolytic and potent immunogenic properties in multiple acute leukemia models. A peculiar form of programmed cell death involves the induction of adaptive immune responses against the dying cell. This process, commonly referred to as immunogenic apoptosis, is essential to the efficacy of several current chemotherapeutics24 and is required for host defense against viral infection29 including live RVs.30 Our in vivo results indicate that a similar process is induced by NRRPs and is a driving factor for treatment efficacy.

More relevant are the observations that multidrug-resistant primary myeloblasts from patients with CML in acute blast crisis are forced into apoptosis and finally eradicated by NRRP treatment. In addition, non-leukemic white cells procured from healthy bone marrow were not adversely affected. This observation suggests that despite the potent tumoricidal activity of NRRPs, the leukopenia commonly observed after standard induction and consolidation chemotherapy could be avoided by using NRRP-based regimens. This would likely significantly decrease treatment-related adverse events. Further, given the preservation of normal white blood cells during leukemic cytoreduction by NRRPs, the simultaneous induction of an effective antileukemic immune response may be attainable for the majority of patients who are not candidates for high-dose radiochemotherapy and HSCT. With the induction of immunogenic apoptosis by NRRPs, a broad array of immunomodulatory cytokines are released by the recipient’s intact immune system, and this likely contributes to development of the effective adaptive antitumor immune activity—a critical component to achieving durable curative responses.

This work demonstrates a feasible biotechnology that produces high-titer NRRPs—an essential requirement for wide-scale clinical advancement. We are currently validating the MTD of NRRPs in several animal models, and are developing good laboratory practice safety measures to ensure stringent confirmation of the non-replicating nature of these bioactive particles. We expect that the pathway to approval should be less onerous than current live RV platforms under development by our laboratory and others. This promising multimodal therapeutic platform is poised for early-phase clinical trials.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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