

## Research Paper

# Anti-tumor activity mediated by protein and peptide transduction of HIV viral protein R (Vpr)

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**Abbreviations:** PTD, protein transduction domain; Vpr, viral protein R; Tat, transactivator of transcription; CP, control peptide; P17, peptide 17; P18, peptide 18; P19, peptide 19; P20, peptide 20

**Key words:** HIV-1, Vpr, protein transduction domain, tat, peptide, cytotoxicity, apoptosis, tumor cells, melanoma

Peptides that are capable of traversing the cell membrane, via protein transduction domains (PTDs), are attractive either directly as drugs or indirectly as carriers for the delivery of therapeutic molecules. One such PTD, a HIV-1 Tat derived peptide has successfully delivered a variety of “cargoes” including proteins, peptides and nucleic acids into cells. There also exists other naturally occurring membrane permeable peptides which have potential as PTDs. Specifically, one of the accessory proteins of HIV (viral protein R; i.e., Vpr), which is important in controlling viral pathogenesis, possesses cell transduction domain characteristics. Related to these characteristics, Vpr has also been demonstrated to induce cell cycle arrest and host/target cell apoptosis, suggesting a potential anti-cancer activity for this protein. In this report we assessed the ability of Vpr protein or peptides, with or without conjugation to a PTD, to mediate anti-cancer activity against several tumor cell lines. Specifically, several Vpr peptides spanning carboxy amino acids 65–83 induced significant (i.e., greater than 50%) *in vitro* growth inhibition/toxicity of murine B16.F10 melanoma cells. Likewise, in *in vitro* experiments with other tumor cell lines, conjugation of Vpr to the Tat derived PTD and transfection of this construct into cells enhanced the induction of *in vitro* apoptosis by this protein when compared to the effects of transfection of cells with unconjugated Vpr. These results underscore the potential for Vpr based reagents as well as PTDs to enhance anti-tumor activity, and warrant further examination of Vpr protein and derived peptides as potential therapeutic agents against progressive cell proliferative diseases such as cancer.

## Introduction

The limited efficacy of many conventional strategies for the treatment of specific cancers warrants the development of novel and more effective drugs. A novel approach being pursued is to exploit viral specific gene products as a new class of potential therapeutics.<sup>1</sup> This strategy is based on the premise that specific viral proteins, even when separated from the other viral replicative machinery and proteins, can retain potent biological activity that may be redirected and utilized for therapeutic purposes. A protein being evaluated for such purposes is the HIV accessory protein Vpr (viral protein R), a 96 amino acid, 15 kDa protein which is important for HIV pathogenesis.<sup>2</sup> The potential therapeutic utility of Vpr is based on its ability, as a free protein, to arrest cells in the G<sub>2</sub>/M phase of the cell cycle, and stimulate rapidly dividing cells to undergo apoptosis.<sup>3-7</sup> In addition, Vpr has been demonstrated to inhibit *in vitro* tumor cell growth of several varied tumor lines, including those of human origin.<sup>8-14</sup> Also, other studies have demonstrated that this anti-tumor activity of Vpr is independent of the p53 expression status of the tumor cells.<sup>15</sup> It has been further indicated that Vpr appears to preferentially inhibit the growth of rapidly dividing and transformed cells as opposed to slowly replicating non-transformed cells which further suggests that this protein may have a useful therapeutic utility against specific cancers without significant toxicity against normal cells.<sup>16,17</sup>

A potential obstacle for therapeutic proteins, including those with anti-cancer activity, is effective delivery of these molecules.<sup>18</sup> Typically, transmembrane delivery/passage of therapeutic agents, including proteins, either through receptor specific or non-receptor mediated endocytosis, is usually required to manifest biological activity. Biological membranes represent major barriers between extracellular and intracellular environments that maintain the critical role of cellular homeostasis. The hydrophobic properties of plasma membranes typically restrict the passage of hydrophilic and charged molecules into and out of cells and limits the therapeutic transport of many biological agents, including proteins and peptides.

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Interestingly, Vpr possesses some characteristics which allow it to effectively transduce (i.e., traverse) across cell membranes. Specifically, carboxy regions of Vpr encompassing a pair of H (F/S) RIG sequence motifs (residues 71–75 and 78–82) have been demonstrated to cause cell membrane permeabilization, resulting in membrane leakage and death in yeast and dividing mammalian cells.<sup>11,19,20</sup> Once in the cytoplasm, Vpr appears to bind to a set of cellular receptors including hVIP/Mov34 and translocates them to the nuclear membrane and, as such, may be involved in the pathway for cell cycle arrest at the G<sub>2</sub> checkpoint.<sup>21,22</sup> This innate ability to traverse the cell membrane provides the Vpr protein with advantages as a potential protein-based drug. Based on these observations, our group initiated some investigations to test whether short Vpr peptides from this carboxy region were able to induce *in vitro* cytotoxicity against the B16.F10 murine melanoma tumor cell line. However, it was unclear if the delivery of Vpr peptides are as efficient as that previously reported for other proteins which possess highly effective PTD characteristics, such as Tat from HIV-1.<sup>23</sup> Based on the considerable evidence for the powerful PTD characteristics of a Tat peptide, which encompasses amino acids 47–57 of Tat, we constructed a conjugate with this peptide and the Vpr protein and tested this reagent for the induction of *in vitro* apoptosis/cytotoxicity in a number of tumor cell lines. The goal of this analysis was to compare the biological activity of unconjugated Vpr protein to that of the Vpr-Tat peptide conjugate. Apart from HIV-1 Tat, other PTDs have been characterized from other sources including the *Drosophila antennapedia* (Antp) and the herpes simplex virus VP22 proteins.<sup>24,25</sup> The Tat peptide was chosen for the conjugation experiments reported here since it had been indicated, from the literature, to be the most extensively characterized and potent of the PTD molecules and can effectively enter cells when added exogenously to culture medium.<sup>26-30</sup>

Finally, the over-arching impetus for the studies reported here is based on the rationale that the generation and characterization of small peptide reagents from the Vpr protein could have therapeutic advantages over the use of either the whole native recombinant protein or plasmids expressing Vpr. Such potential advantages include cost and production issues as well as better specificity and affinity for targets.<sup>31,32</sup> The effective use of PTD conjugation could potentially increase the therapeutic index of Vpr by further enhancing transmembrane delivery. This is relevant since the Vpr delivery systems employed to date, i.e., through intratumoral electroporation of naked DNA plasmids or virally based expression vectors, have been somewhat inefficient which could account for the minimal to moderate therapeutic index currently observed with these reagents.<sup>12,13,15</sup>

## Results

**In vitro peptide mediated cytotoxicity of B16.F10 murine melanoma cells.** Based on the previously presented rationale, we examined the potential biological effects of incubation of B16.F10 cells with carboxy region Vpr peptides. It was observed that several peptides from this carboxy region of Vpr mediated inhibition of tumor cell proliferation. The results of these analyses are expressed as either mean optical density (at OD570 nm) values from the MTT dye assay (Fig. 1A and C) or percent inhibition of proliferation compared to control peptide (i.e. CP) (Fig. 1B and D). The inhibition at the 16 hr post-treatment time point appeared to demonstrate a dose-response

relationship (Fig. 1A and B) with the higher dose resulting in greater suppression of cell viability. At this time point, the percent inhibition induced (compared to the CP) was significantly greater (i.e., at  $p < \text{or equal to } 0.5$  as determined by a statistical non-parametric Mann-Whitney U test) for P17 and P18 at both the 25  $\mu\text{M}$  and 50  $\mu\text{M}$  concentrations (Fig. 1B). Specifically, the percent inhibition at 25 or 50  $\mu\text{M}$  induced by P17 was 36% or 58% respectively. For P18 the percent inhibition at concentrations of 25 or 50  $\mu\text{M}$  was 41% and 56% respectively. For P19, significant inhibition was noted at the 50  $\mu\text{M}$  (i.e., 35%) but not with the 25  $\mu\text{M}$  (i.e., 14%) concentration. In contrast, at the 40 hour post-incubation time point, (Fig. 1C and D) significant inhibition (i.e., at  $p < \text{or equal to } 0.5$  as determined by a non-parametric Mann-Whitney U test) was noted only with the 50  $\mu\text{M}$  concentrations of P17 (i.e., 60%), P18 (i.e., 41%) or P19 (i.e., 27%). Likewise, inhibition of B16.F10 cell proliferation was not noted with incubation with P20, at the concentrations or time points tested in this study. Also, statistical analysis by the Mann Whitney U test indicated that incubation of cells with 50  $\mu\text{M}$  of P17 resulted in significantly greater cytotoxicity than the 25  $\mu\text{M}$  concentration of this peptide ( $p < \text{or equal to } 0.5$ ). Analysis of the cytotoxicity assay by Trypan Blue exclusion and the WST-1 reagent provided analogous results (data not shown) to the MTT dye assay.

These experiments indicated that inhibition of cell proliferation decreased progressively as the peptides tested were more proximal toward the carboxy terminus, with the most carboxy peptide (P20) failing to mediate any biological activity. Inhibition with 50  $\mu\text{M}$  of P17 was maintained at 40 hrs when compared to the 16 hr incubation time point. At the 40 hr time point other peptides tested (i.e., P18 and P19) were not as potent at the 50  $\mu\text{M}$  concentration compared to the 16 hr time point with significant inhibition disappearing for all these peptides at the 25  $\mu\text{M}$  concentrations.

**Generation of pVpr and pVpr-PTD and analysis of the induction of apoptosis in tumors cells transfected with these constructs.** Previous studies have indicated that Vpr alone can penetrate fairly effectively into cells without the need for carriers or receptor-targeting strategies.<sup>45,46</sup> This is further supported by the Vpr peptide inhibition of B16.F10 cell proliferation presented above. Additionally, the natural transducing properties of Vpr indicated that this protein is an excellent candidate for the evaluation of the potential enhancement of cell entry/penetration thru conjugation to an established PTD. As such, we hypothesized that Vpr, when combined (i.e., conjugated) with additional transduction inducing “help” from a PTD would exhibit cooperatively higher tumor cell killing activity, presumably through mediating more efficient translocation of the protein across the cell membrane.

To test this hypothesis, a pVpr-PTD plasmid, encoding for Vpr conjugated to the tat PTD, was cloned and expressed (Fig. 2) as described in the Materials and Methods section. Specifically, Figure 2A indicates the pVax plasmid backbone which expresses Vpr-PTD under the control of a CMV promoter. Subsequently, the correct size of the DNA for the Vpr-PTD conjugate was confirmed by standard agarose gel electrophoresis (Fig. 2B), in which identical samples were electrophoresed in lanes 1 and 2 of the gel. In addition, protein expression from the Vpr-PTD and pVpr alone plasmids was confirmed by an *in vitro* transcription/translation reaction followed by immunoprecipitation with a specific anti-Vpr antibody and SDS-PAGE (Fig. 2C). These analyses validated both the Vpr-PTD

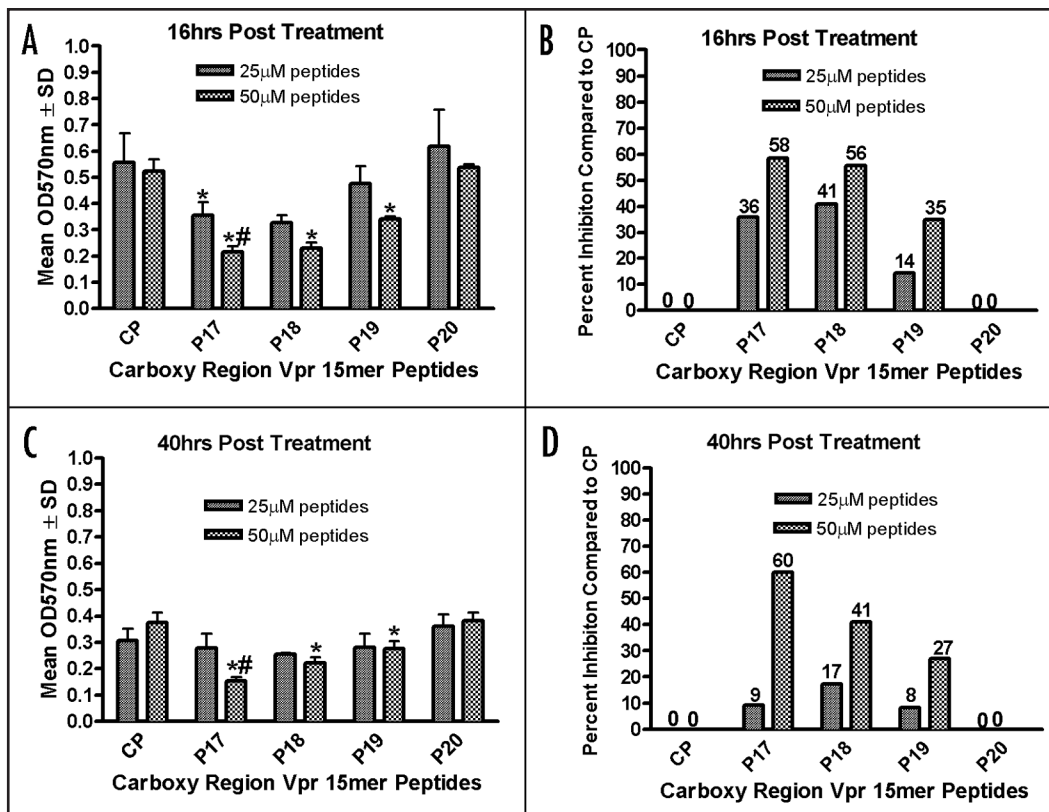


Figure 1. In vitro cytotoxicity of B16.F10 murine melanoma cells induced by carboxy region Vpr peptides. In vitro peptide induction of cytotoxicity is indicated in this Figure by MTT dye assay OD570 nm values plotted versus different concentrations of the tested peptides. The graphs indicating these data for the 16 and 40 hour time points are shown in (A and C) respectively. (B and D) indicate the percent inhibition of proliferation (i.e., cytotoxicity) of the tumor cells by the Vpr peptides compared to a control peptide (CP) at the 16 and 40 hour time points respectively and are based on the MTT dye assay values presented in (A and C). The amino acid sequences for the peptides tested are presented in Table 1. The asterisks (\*) indicated on some of the graph columns indicate statistically significant inhibition (at  $p < \text{or equal to } 0.05$  level using a non-parametric Mann Whitney U test) of B16.F10 proliferation by individual Vpr peptides when compared to equivalent concentrations of CP. As well, for P17, non-parametric Mann Whitney U test analysis indicated that the 50  $\mu\text{M}$  concentration of peptide (at  $p < \text{or equal to } 0.5$ ) induced cytotoxicity to a significantly greater extent than the 25  $\mu\text{M}$  concentration. This is shown in (A and C) using a # symbol on the appropriate columns indicating the 50  $\mu\text{M}$  concentrations for P17.

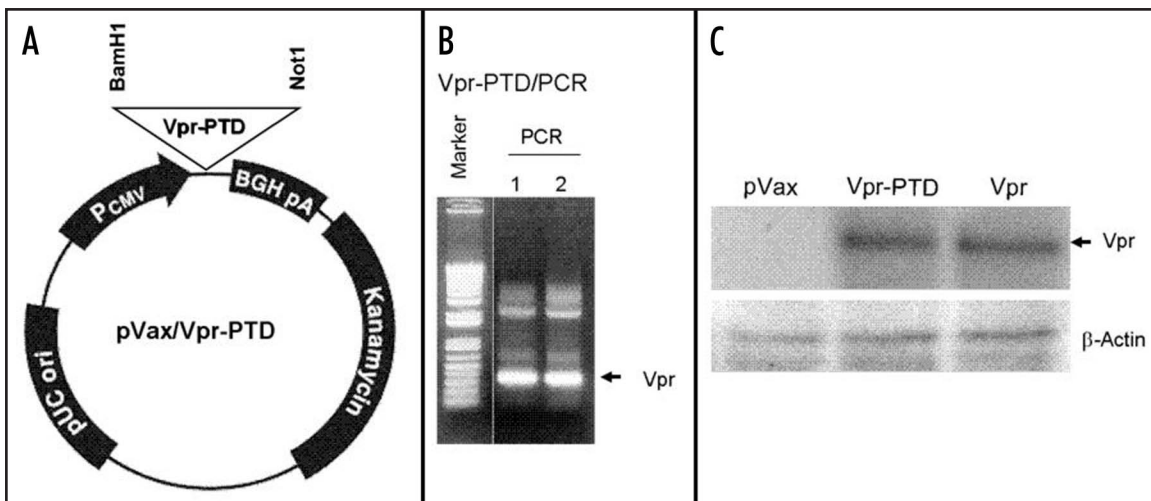


Figure 2. Construction and expression of the Vpr-PTD plasmid. (A) Schematic representation of Vpr-PTD plasmid construction. (B) DNA from PCR samples of the pVpr-PTD constructs were analyzed (duplicate identical samples in lanes 1 and 2) by 1% agarose gel electrophoresis. The arrow indicates the Vpr gene insert in lanes 1 and 2. (C) Expression of the Vpr-PTD plasmid. Plasmids (1  $\mu\text{g}$ ) were used for coupled in vitro transcription/translation reactions. Immunoprecipitation of the in vitro-translated proteins was performed with an anti-Vpr polyclonal antibody. The immunoprecipitated proteins were eluted from the Sepharose beads and subjected to SDS-PAGE using 12% gels and processed for fluorography as described.<sup>38</sup> The arrow indicates the expression of the Vpr protein.

and unconjugated Vpr expressing constructs for use in the subsequent experiments.

Using the pVpr and pVpr-PTD plasmids generated, we determined whether an increased tumor-killing effect (i.e., apoptosis) by Vpr-PTD, compared to unconjugated Vpr, occurred after transfection of the plasmids into a range of different tumor cell phenotypes (HeLa, SK-N-AS, TE671, 1ZR75-1, THP-1 and LnCap). The type/derivation of these tumor lines are listed in the Material and Methods section. Seventy-two hours after transfection with either pVpr or pVpr-PTD, cells were collected and stained with Annexin-V-FITC in order to measure specific Vpr or Vpr-PTD induced apoptosis. Importantly, as the data presented in the Figure indicates, transfection of the cells with the pVax backbone vector did not induce significant cytotoxicity (peak 1). Specifically levels of apoptosis in the cell lines were as indicated in Figures 3A (HeLa), B (SK-N-AS), C (TE671), D (ZR75-1), E (THP1) and F (LnCap). In all of the cell lines tested, transfection with the pVpr-PTD construct resulted in higher levels of apoptosis compared to cells transfected with pVpr alone. This is indicated by the relative position and size of the peaks (mean fluorescence intensity = MFI) which are designated 2 (pVpr) and 3 (pVpr-PTD) in the individual panels of Figure 3. Specifically, the ratio of the MFI of peak 3 to peak 2 ranged from 1.3 for HeLa cells to 2.7 for TE671 cells. The results demonstrate that conjugation of Vpr to a PTD significantly increases the level of induction of apoptosis in all of the varied tumor cell types tested in this study.

## Discussion

Conventional approaches for the delivery of pharmacological agents, including anti-cancer agents, often suffer from poor target specificity and unwanted side effects (i.e., toxicity against normal cells and tissues). Gene therapy with viral vectors can also be problematic because of their potential to induce adverse immune responses as has been observed in some recent human clinical trials.<sup>47</sup> Therefore, protein transduction domain delivery of “cargo”, which functions without reliance on specific cell surface receptors, is a potentially attractive option for drug delivery and therapy.<sup>48-52</sup> The HIV-1 accessory protein Vpr (viral protein R) has been demonstrated to possess cell transducing (i.e., functions as a natural PTD) characteristics which allow it to cross cell membranes without carriers or receptor-targeting strategies.<sup>19,49,51</sup> This protein, which has an important role in HIV pathogenesis, also has been demonstrated to induce cell cycle inhibitory and pro-apoptotic activity that is potentially useful for cancer therapy.<sup>15,51,52</sup> In fact, several studies have demonstrated *in vitro* as well as *in vivo* inhibition of several tumors by the Vpr protein.<sup>8,9,11,14,50,53,54</sup> We have recently reported the *in vivo* anti-cancer activity of electroporation enhanced delivery of a DNA plasmid expressing Vpr against *in vivo* established B16.F10 murine melanoma tumors.<sup>12,13</sup> In these experiments, a small but statistically significant percentage of mice underwent complete regression of their established subcutaneous tumors after treatment. This tumor regression effect is thought to be biologically significant, and potentially applicable to human tumors, since the B16.F10

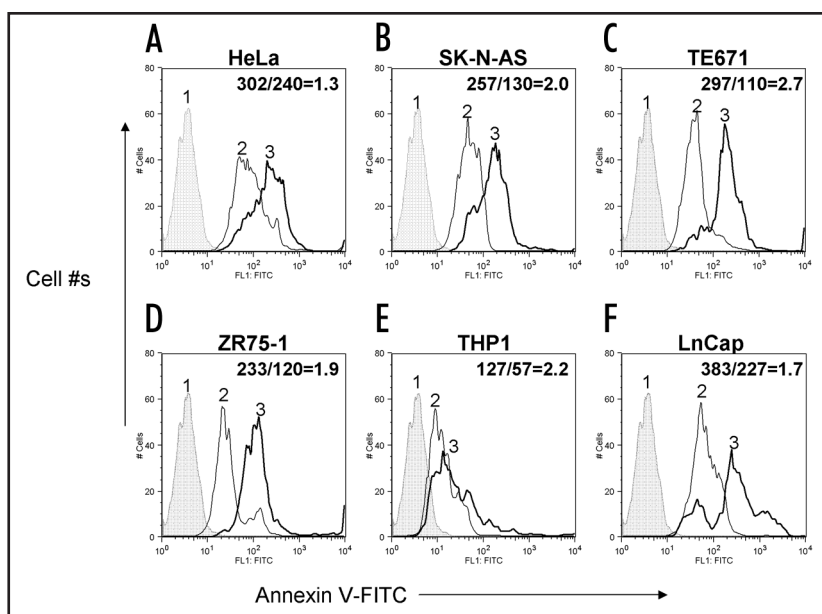


Figure 3. *In vitro* induction of apoptosis in different tumor cell type lines by Vpr or Vpr-PTD. Apoptosis was measured in six different tumor cell lines transfected with either pVpr or pVpr-PTD plasmid followed by staining with Annexin V-FITC at 72 hours post transfection. The cell lines transfected are indicated in the six panel graphs (A through F) with the tumor types listed in the Materials and Methods section. The numerical designations for the peaks (indicating the different plasmids for transfection) are as follows: 1 = pVax control, 2 = pVpr and 3 = pVpr-PTD. In each of the panels the numerical values given are the mean fluorescence intensity (MFI) for pVpr (peak 2) and pVpr-PTD (peak 3), expressed as a ratio (peak 3/peak 2). The data presented are representative of two independent experiments.

tumor cell line is highly aggressive, metastatic and poorly immunogenic.<sup>55-57</sup> In addition, very few other therapeutic strategies have proven to be successful in inducing regression of established tumors generated from B16.F10 cells.<sup>58</sup> These observations provided further experimental support for the potential utility of Vpr as an anti-cancer agent and the impetus for further analysis, by our group, of the biological activity of this protein.

In this report, we investigated the anti-tumor potential of Vpr based peptides. In addition, we assessed the effect of combining the inherent cell membrane transducing properties of Vpr with additional “help” from a highly characterized PTD from HIV-1 Tat which was generated by cloning and expressing the two sequences together in a Vpr-Tat PTD plasmid conjugate.<sup>59</sup> As indicated, the rationale for these experiments was based on an interest to develop and evaluate the anti-tumor potential of other Vpr based reagents. Previous studies have utilized, by us as well as others, either protein or DNA based Vpr reagents. The DNA based reagents were, as indicated, either delivered through live viral vectors or naked DNA plasmids via *in vivo* electroporation.<sup>9,12,13,15,59,60</sup> As indicated, these experiments demonstrated some anti cancer activity for Vpr, albeit at a low to moderate level. Therefore, we reasoned that other investigations were warranted to evaluate other Vpr based reagents (i.e., peptides or PTD conjugated protein) in terms of potential anti-cancer activity.

In the Vpr peptide experiments reported here, potential inhibition of *in vitro* B16.F10 cell proliferation was quantitated using several

carboxy region Vpr peptides spanning amino acids 65 through 91. The results indicated that peptides derived from the most amino segments (i.e., P17 and P18) of the carboxy region peptides tested, mediated a dose related significant inhibition of B16.F10 melanoma tumor cell proliferation *in vitro*. As additional peptides were tested which were more carboxy within this region (i.e., peptides P19 and P20), inhibition of tumor cell proliferation was progressively decreased. These results, summarized in Figure 1, indicated that the highest level of inhibition of B16.F10 cell proliferation was mediated by peptide segments spanning amino acids 65–83. All of the peptides tested contained a motif, designated H (F/S) RIG, which has been previously identified as mediating the penetration of Vpr into CD4<sup>+</sup> lymphocytes with concomitant induction of mitochondrial dysfunction and apoptosis.<sup>61</sup> Although all of the peptides contained this motif, not all of these peptides mediated equivalent inhibition of B16.F10 proliferation. In fact, as indicated above, the more carboxy of these peptides, within the 65–91 amino acid region, inhibited proliferation progressively less than the more amino fragments. This observation suggests, at least in these studies, that regions within the peptides other than the established H(F/S)RIG motif also have a functional role in the *in vitro* inhibitory effects noted against tumor cells. Future studies can likely resolve this issue through the use of other control peptides as well as Vpr peptides P17 through P19 which contain appropriate amino acid deletion/substitutions.

In addition to examining the effects of unmodified Vpr peptides on tumor cell proliferation, we also decided to examine the anti-tumor activity of Vpr conjugated to the HIV-1 Tat PTD peptide. This was based on the relatively large body of evidence validating the effectiveness of PTDs to mediate and enhance cell membrane penetration of a variety of “cargoes”. As indicated, PTDs, such as the Tat peptide and the *Drosophila antennapedia* transduction domain, have been studied extensively and, in particular, for applicability to cancer therapy. One study dealt with the tumor suppressor gene p53 which is mutated in 50% of all human cancers. The loss of normal p53 function typically increases the resistance of cancer cells to subsequent therapy.<sup>62</sup> Attachment of the C-terminal p53 peptide to the *Drosophila antennapedia* transduction domain, and delivery of this conjugate to tumor cell lines, was found to induce apoptosis in these cells.<sup>63</sup> Significantly, normal cells, which express wild type p53, were not affected by the transduction of the exogenous p53 peptide. Likewise, *in vivo* studies using p53 fused to Tat PTD reported similar anti-cancer efficacy.<sup>64,65</sup> Another example of the potential clinical efficacy of PTDs was a study involving the tumor suppressor gene p16<sup>INK4</sup> which is frequently functionally inactivated in human cancers (e.g., melanoma, pancreatic adenocarcinomas, lung cancers, etc.).<sup>66</sup> In these cells, covalent linkage of p16 peptide to the Tat transduction domain resulted in intracellular accumulation of p16, leading to inhibition of cyclin-D: cdk4/6 activity and eventual G<sub>1</sub> arrest of the transformed cells.<sup>67</sup> Moreover, a recent report has demonstrated the induction of apoptosis and inhibition of growth of several melanoma cell lines when a survivin antagonist-Tat PTD conjugate was delivered to the cells.<sup>68</sup> Importantly, other studies have indicated that the Tat derived PTD alone does not induce significant cytotoxicity. Specifically, Vives and colleagues demonstrated that two Tat peptides spanning amino acids 43–60 and 48–60, both of which contain the 11 amino acid PTD used in the study reported here, were not able to mediate significant cytotoxicity when incubated for

extended intervals at a concentrations of 100 μM.<sup>69</sup> In summary, the growing list of deliverable therapeutic peptides and proteins suggests that protein transduction therapy will likely become an emerging modality in the field of cancer therapeutics in the near future.

Based on the attractiveness of the PTD technology, we tested the conjugation of Vpr to the Tat-PTD and assessed the specific induction of apoptosis in different tumor cell lines after transfection with the Vpr and Vpr-PTD plasmids. The data from these experiments indicated that PTD conjugation to Vpr significantly enhanced the level of apoptosis induction in a number of different tumor cell lines suggesting a broad applicability of activity against a range of cancer cell types.

In summary, these studies underscore the potential of unmodified and PTD conjugated Vpr peptides/proteins as therapeutic agents against cancer. To our knowledge this is the first report demonstrating the ability of Vpr peptides, or Vpr protein conjugated to a PTD, to mediate *in vitro* tumor cell cytotoxicity/apoptosis. Future studies will include further mechanistic analysis as well as assessment of *in vivo* anti-tumor activity of these novel Vpr based reagents, when delivered by targeted and systemic routes. In addition, more recent studies have suggested that other domains of Vpr may molecularly mimic the activity of other established anticancer agents.<sup>70</sup> As such, a more extensive analysis of other regions of Vpr are planned to assess their potential anticancer activity.

## Materials and Methods

**Cell lines, peptides and proteins.** HeLa<sup>33</sup> (human cervical carcinoma), SK-N-AS<sup>34</sup> (human neuroblastoma), TE671,<sup>35</sup> (human medulloblastoma), ZR75-1,<sup>36</sup> (human breast carcinoma), THP1,<sup>37</sup> (human acute monocytic leukemia), LnCap<sup>38</sup> (androgen-sensitive human prostate adenocarcinoma) and B16.F10,<sup>39</sup> (murine melanoma) tumor cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The HeLa, TE671 and SK-N-AS cell lines were grown in a Dulbecco's Modified Eagle's medium while the murine B16.F10 melanoma cell line was cultured in McCoy's 5A medium. In addition, THP1 cells were cultured in MEM medium while ZR75-1 and LnCap cells were grown in RPMI1640 medium. All the growth media was supplemented with 10% fetal bovine serum (BSA) as well as specific additives, where appropriate. As well, all of the cell lines were cultured and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

The Vpr peptides (15 amino acids in length) tested in the *in vitro* assays against B16.F10 cells were obtained from the AIDS Research and Reference Reagent Program (Germantown, MD). Stocks of the peptides used in experiments were reconstituted to 1 mg/ml in distilled/deionized water. The peptides tested, with their designations and amino acid numbers and sequences are listed in Table 1. As indicated, peptides 17, 18, 19 and 20 are designated P17, P18, P19 and P20 respectively. The control peptide (CP) used in these experiments was derived from the envelope glycoprotein of SIV and had the following amino acid sequence: EQEQMISCKFNMTGL. This peptide was used as a control in these studies since it lacked any established PTD characteristics or biological activity against the murine melanoma cells used in this study.

**Assessment of Vpr peptide mediated cytotoxicity.** Peptide induced cytotoxicity was assessed by several different methodologies. The first was the standard Trypan blue dye exclusion assay which uses 0.4% Trypan blue mixed with cells in which exclusion of the

dye indicates viable cells. The other more quantitative measurements are by the MTT (3-(4, 5-dimethyliazol-2-yl)-2, 5-diphenyl tetrazolium bromide) or WST-1 (4-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5 tetrazolio-1-3-benzene disulfonate) tetrazolium based dye reduction assays.<sup>40,41</sup> The methods for the MTT (Roche Diagnostics, GmbH (Penzberg, Germany) and WST-1 (Promega Corporation, Madison, WI) dye reduction assays were followed as described by the manufacturer.

For the peptide cytotoxicity assays the following methodology was utilized: Peptides tested were diluted from stocks to the appropriate molar concentrations for evaluation in in vitro studies against the B16.F10 cells. Actively growing B16.F10 cells were harvested and resuspended in fresh media to  $5 \times 10^5$  cells/ml. Cells ( $100 \mu\text{l} = 5 \times 10^4$  cells) were then subsequently mixed with peptides at final concentrations of either 25 or 50  $\mu\text{M}$  and incubated in 96 well plates at 37°C at 5% CO<sub>2</sub> for either 16 or 40 hours. The selection of the 16 and 40 hr incubation/measurement time intervals was based on the scientific literature indicating that the approximate doubling time of the B16.F10 melanoma cell line is between 16 and 23 hrs.<sup>42</sup> Therefore, it was reasoned that the incubation/measurement time intervals selected for this study would encompass an approximate 1–2 doublings of in vitro growing untreated B16.F10 tumor cells. As such, measurement at these intervals should allow for adequate and instructive assessment of any biological activity resulting from the peptide treatments. At the 16 and 40 hour time points the B16.F10 cell plus peptide mixtures were assessed for cytotoxicity by the assay methods described above, i.e., the Trypan blue exclusion method or the spectrophotometric tetrazolium based measurements (MTT or WST-1 dye assays).<sup>40,41</sup> At these time points cytotoxicity was assessed by measuring absorbance in the wells at OD (optical density) of 570 or 450 nm for the MTT or WST-1 assay respectively. The level/extent of cytotoxicity in wells incubated with cells and experimental peptides was compared to wells incubated with the control peptide (CP). Appropriate statistical analysis (i.e., non-parametric Mann-Whitney U test) was performed to determine any significant differences between cytotoxicity induced by the CP and the experimental peptide groups. The levels of statistical significance was at the 5% level (i.e.,  $p < \text{or equal to } 0.05$ ).

**Construction and expression of the Vpr-PTD plasmid.** The Vpr plasmid was constructed by cloning the Vpr gene/protein using the following primer pairs Vpr (F): ACG GAT CCA TGG AAC AAG CCC CAGA and Vpr(R) TGG ATC TAC TGG CTC CATT. For construction of the Vpr-PTD conjugate the PTD-Tat sequence (encoding for amino acids YGRKKRRQRRR encompassing residues 47–57) was attached to the 3'/C-terminus of the gene for Vpr followed by cloning of the amplified PCR product (via BamH1 and Not1 restriction sites) into the pVax1 vector (Invitrogen, CA) and sequenced to verify the Vpr gene.

The successful generation of the pVax/Vpr-PTD construct was confirmed by 1% agarose gel electrophoresis analysis of identical samples of the reaction mixture and the expression was subsequently confirmed by a method using the TNT-coupled in vitro transcription/translation system (Promega Corp., WI) in which <sup>35</sup>S-labeled protein products were generated using plasmids containing 1  $\mu\text{g}$  of pVpr (wild type), or pVpr-PTD. The reaction mixture was prepared according to the instructions supplied by the manufacturer and was carried out at 30°C for one hour as described previously.<sup>43</sup> Subsequently, 25  $\mu\text{l}$  of in vitro-translated <sup>35</sup>S-labeled proteins were pre-cleared with protein G-Sepharose four Fas Flow beads (Amersham Bioscience, NJ) and

**Table 1 Carboxy region 15 amino acid Vpr peptides used in study**

Peptide designation	Amino acid #	Amino acid sequence
17 = P17	65–79	QQLLFHFHFRIGCQHS
18 = P18	69–83	FIHFHFRIGCQHSRIGI
19 = P19	73–87	RIGCQHSRIGITQQR
20 = P20	77–91	QHSRIGITQQRARRN

P17 thru P20 are the designations for the peptides tested in the in vitro assays. The amino acid sequences and the range of residue numbers within the full length Vpr protein are provided for each of the peptides.

goat IgG (Santa Cruz Biotechnology, CA) at 4°C for one hour. The Sepharose beads were washed four times with 5 ml of protein lysis buffer containing 0.5 M NaCl and once more with protein lysis buffer. Proteins were immunoprecipitated using a 1:1000 dilution of anti-Vpr polyclonal antisera (obtained from the NIH AIDS Research & Reference Reagent Program, MD). Approximately 5 mg of protein G-Sepharose beads were subsequently added to each immunoprecipitation reaction and the samples were incubated at 4°C for 90 min in a rotating shaker. The beads were then subsequently washed 3 times with binding buffer containing high salt and bovine serum albumin (BSA) and finally suspended in 2X SDS sample buffer. The immunoprecipitated protein complexes were eluted from the Sepharose beads by briefly boiling and were then resolved on 12% SDS-PAGE gels. The gel was subsequently fixed, treated with a 1 M sodium salicylate solution, and dried in a gel drier (Bio-Rad, CA). The dried gel was exposed overnight to X-ray film and developed using an automated developer (Kodak, NY).<sup>43</sup>

**Apoptosis in different tumor cell lines induced by Vpr or Vpr-PTD constructs.** The six different tumor cell lines used in this study were selected since they represent a range of different tumor types. Briefly, the cells were transfected ( $10^6$  cells each) with either 5  $\mu\text{g}$  of the control backbone (pVax), pVpr or pVpr-PTD plasmids. Specifically, tumor cells ( $10^6$  per well) were seeded in 60-mm plates 24 hours before transfection (i.e., at 60–80% cell confluency). Transfections were conducted with the FuGENE 6 transfection reagent (Roche Applied Science, Nutley, NJ) according to the manufacturer's instructions with some modifications. Total amounts of DNA were maintained constant in all reaction mixtures by using backbone control vectors. The transfection efficiency in these cells was approximately 45–50% as measured using a green fluorescent protein vector (pEGFP, Clontech, CA) by fluorescence-activated cell sorter (FACS) analyses of GFP expression. The expression attained using this methodology had previously been demonstrated to be stable for at least as long as the experimental incubation time of the transfected cells in these studies (i.e., 72 hours). Apoptosis was assessed in transfected cells according to the following methods:<sup>44</sup> Seventy-two hours post transfection, cells were collected and stained with Annexin V-FITC to measure Vpr induced apoptosis in order to assess any differences between pVpr and pVpr-PTD. Annexin V-FITC staining was performed by manufacturer's recommended methods using a kit from BD Pharmingen (San Diego, CA). Briefly, FACS analysis was performed on a gated low forward scatter and side scatter for Annexin V positive cells, which were indicative of apoptosis.

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