[CANCER RESEARCH 59, 4200–4203, September 1, 1999]

Advances in Brief

The Addition of Adenovirus Type 5 Region E3 Enables Calydon Virus 787 to Eliminate Distant Prostate Tumor Xenografts

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Abstract

CV787, a novel highly prostate-specific replication-competent adenovirus with improved efficacy, was constructed. CV787 contains the prostate-specific rat probasin promoter, driving the adenovirus type 5 (Ad5) *E1A* gene, and the human prostate-specific enhancer/promoter, driving the *E1B* gene. To improve efficacy, we constructed CV787 such that it also contains the entire Ad5 E3 region. CV787 replicates in prostate-specific antigen (PSA)$^+$ cells as well as wild-type adenovirus, but in PSA$^-$ cells, CV787 replicates 10^4–10^5 times less efficiently. CV787 destroys PSA$^+$ prostate cancer cells 10,000 times more efficiently than PSA$^-$ cells. Incorporation of the Ad5 E3 region significantly improves the target cell killing ability or efficacy of CV787. In *nu/nu* mice carrying s.c. LNCaP xenografts, a single i.v. tail vein injection of CV787 eliminates 300-mm^3 tumors within 4 weeks. CV787 could be a powerful therapeutic for human metastatic prostate cancer.

Introduction

Prostatic cancer is the second leading cause of cancer-related deaths in men in the United States (39,000 deaths in 1998; Ref. 1). Current treatment for metastatic prostate cancer is androgen ablation therapy, which, in 70% of men, provides relief from otherwise uncontrollable bone pain and increases life expectancy by 6–18 months. Clearly, a new approach to metastatic prostate cancer treatment is needed (2–4).

We previously constructed a prostate-specific ARCA,[2] CN706, in which the Ad5 *E1A* gene is driven by PSE (5, 6). CN706 destroys human PSA$^+$ cells 400 times more efficiently than PSA$^-$ cells and eliminates LNCaP xenografts in *nu/nu* mice with a single i.t. injection. To improve the specificity of CN706, we placed both the Ad5 *E1A* and *E1B* genes under the control of prostate-specific transcriptional regulatory elements. CV764 contains the PSE, driving the Ad5 *E1A* gene, and the promoter/enhancer of a second human prostate-specific gene, the *hKLK2* gene, driving the Ad5 *E1B* gene. CV764 destroys PSA$^+$ cells 10,000 times more efficiently than PSA$^-$ cells and cannot productively infect PSA$^-$ cells (7). However, CN706 and CV764 could not eliminate distant preexistent LNCaP xenograft tumors in *nu/nu* mice by i.v. tail vein administration (data not shown).

To improve efficacy by systemic i.v. administration, we, led by preliminary evidence (data not shown), restored the Ad5 E3 region (nucleotides 28133–30818). The E3 region had been deleted in both CN706 and CV764 (6–8). The E3 region has long been considered unnecessary for replication of adenovirus *in vitro* and has been universally deleted from Ad5 gene therapy constructs until recent efforts to reduce the immune response to the vector (9–14). The Ad5 E3 region encodes proteins that play a role in assisting virus release and evading or slowing host immune responses to the virus (15–25). CV764 did not have the genome space required to include the entire E3 region, but we wished to retain the specificity of using two independent prostate-specific transcriptional regulatory elements driving the Ad5 *E1A* and *E1B* genes. Thus, we constructed CV787 using the rat probasin prostate-specific promoter (26–28), driving the *E1A* gene, and the PSE, driving the *E1B* gene, and retained the entire Ad5 E3 region. The CV787 genome length is 105% the length of wt Ad5, yet the virus replicates well and is completely stable. CV787 is an ARCA that replicates like wt Ad5 in cells that express PSA but is attenuated 10,000–100,000 times, with respect to replication in PSA$^-$ cells. CV787 is highly cytopathogenic in PSA$^+$ cells and is capable of eliminating distantly located preexisting prostate tumors following i.v. injection.

Materials and Methods

Virus Constructions. CV787 was constructed by insertion of the rat probasin promoter and the human PSE, driving the Ad5 *E1A* and *E1B* genes, respectively. pXC1, pBHG10, and pBHGE3 were purchased from Microbix Biosystems (Ontario, Canada; Refs. 8 and 29). pXC1 contains the Ad5 bp 22–5790, including the inverted terminal repeat, the packaging sequence, and the *E1A* and *E1B* genes inserted into pBR322 (29). pBHGE3 contains the entire Ad5 genome, except for a deletion between Ad5 bp 188 and 1339, inserted into pBR322 (8). pBHG10 is similar to pBHGE3, except that it is E3 deleted (ΔAd5, nucleotides 28133–30818). pXC1 was modified to contain an *Age*I site at bp 547 between the E1A mRNA cap site and the E1A translation initiation site by inserting a T between Ad5 bp 551 and 552, yielding CP95 (6). CP95 was modified to create an *Eag*I site at Ad5 bp 1681 between the E1B promoter and the E1B mRNA cap site. The *Eag*I site was created by inserting a G between Ad5 bp 1681 and 1682 into CP95 using overlap PCR with the following two sets of primers. The first set [primer i, 5′-TCGTCTTCAA-GAATTCTC-3′; and primer ii, 5′-GCCCA*CGGCCG*CATTATATAC-3′; *Eag*I site is italicized), amplifies a 2090-bp fragment in CP95, and the second set (primer iii, 5′-GTATATAATG*CGGCCG*TGGGC-3′; and primer iv, 5′-CCAGAAAATCCAGCAGGTACC-3′), amplifies a 399-bp fragment from the same plasmid. The two PCR products were annealed in equal molar ratios and used as template for PCR with primers i and iv. The 2468-bp overlap product was digested with *Eco*RI and *Kpn*I and ligated to similarly cut CP95, creating CP124. CP125 was constructed by cloning the PSA 5′-flanking sequence, containing the enhancer domain from −5322 to −3875 from the transcription start site and the promoter from −230 to +7, into the *Eag*I site of CP124 (6). CP257 was constructed by cloning the rat probasin promoter (positions −426 to +28; Ref. 26) into the *Age*I site of CP125. The rat probasin promoter was amplified by PCR from rat genomic DNA (Clontech, Palo Alto, CA) with primers (5′-GATC*ACCGGT*AAGCTTCCACAAGTGCATTTAGCC-3′ and 5′-GATC*ACCGGT*CTGTAGGTATCTGGACCTCACTG-3′) containing *Age*I sites (italicized). The PCR product was digested with *Pin*AI (an isoschizomer of *Age*I) and cloned into a similarly cut CP125, creating CP257. CV739, CV787, and CV802 were generated by homologous recombinations of CP257 and pBHG10, CP257 and pBHGE3, and pXC1 and pBHGE3, respectively (8, 10, 30). Fig. 1 shows the structures of CN702, CN706, CV739, CV787, and CV802. Viruses were grown and purified as described previously (6, 7). The particle:pfu ratio of all viruses was 20:1.

Received 4/21/99; accepted 7/20/99.

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[2] The abbreviations used are: ARCA, attenuated replication-competent adenovirus; Ad5, adenovirus type 5; P, virus particle; PSA, prostate-specific antigen; PSE, PSA promoter and enhancer; i.t., intratumoral(ly); wt, wild-type; pfu, plaque-forming unit(s); MTT, 3-[4,5-dimethylthiazole-2–4]-2,5-diphenyl-2H-tetrazolium bromide.

Fig. 1. Structure of viruses. CN706 contains the PSE, driving the Ad5 *E1A* gene, but is Ad5 E3 region deleted; CV739 contains the rat probasin promoter, driving the Ad5 *E1A* gene, and the PSE, driving the Ad5 *E1B* gene, but is Ad5 E3 region deleted; CV787 is identical to CV739 but contains the Ad5 E3 region; and CV802 is a constructed wt Ad5 containing a normal Ad5 E1 region and a normal Ad5 E3 region.

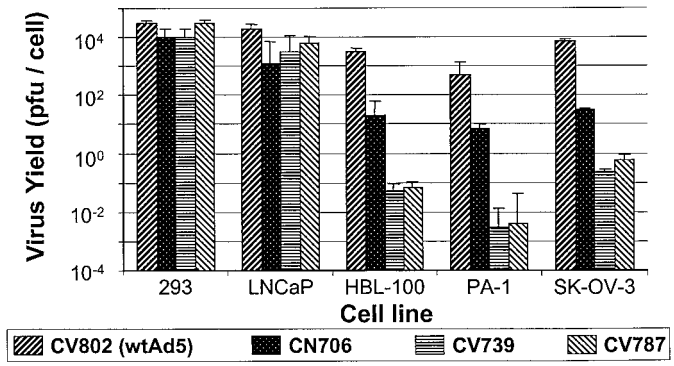

Fig. 2. Burst sizes of CN706, CV739, CV787, and CV802 (wt Ad5) in PSA^+ and PSA^- cells. Cells were infected at a multiplicity of infection of 2, virus was harvested 48 h after infection, and virus yield was determined by plaque assay on 293 cells.

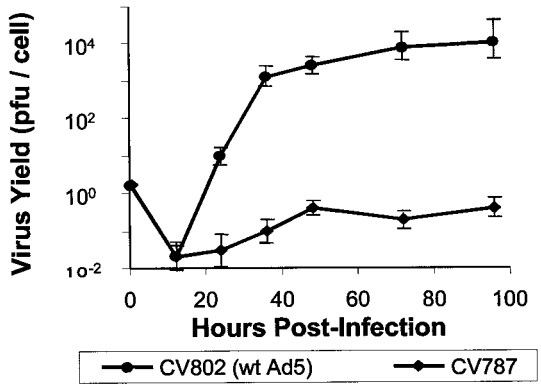

Fig. 3. Growth curves of CV787 and CV802 (wt Ad5) in hMVEC cells. Cells were infected at a multiplicity of infection of 2, virus was harvested at indicated times after infection, and virus yield was determined by plaque assay on 293 cells.

Cell Culture. Cells were obtained and maintained as described previously (6, 7). Plaque assays were determined as described previously (6, 7). Burst sizes of CN706, CV739, CV787, and CV802 (wt Ad5) in PSA^+ and PSA^- cells were determined as follows. Cell monolayers (2×10^5 cells/well) in six-well plates were inoculated with 4×10^5 pfu of CN706, CV739, CV787, or CV802 (wt Ad5; Refs. 7 and 8). Virus yields were titrated at 48 h after infection. Cells were infected in duplicate; assays were carried out in triplicate.

One-step growth curves of CV787 and CV802 (wt Ad5) were performed in human microvascular endothelial cell (hMVEC) monolayers. Monolayers of hMVEC cells were infected at a multiplicity of 2 pfu/cell with either CV787 or CV802 (wt Ad5). At the indicated times thereafter, duplicate cell samples were harvested and lysed by three cycles of freeze-thawing, and the virus in the supernatants was assayed in triplicate in 293 cell monolayers. One-step growth curves of CV787 in LNCaP cells using medium containing charcoal-stripped serum with or without R1881 (methyltrienolone) were performed similarly. Cytopathogenity of CV787 and CV802 (wt Ad5) was also determined in hMVEC monolayers, as described previously (7)

Cell survival of PSA^+ and PSA^- cells infected with either CV787 or CV802 (wt Ad5) was determined. Monolayers of LNCaP, HBL-100, and OVCAR-3 cells were infected at a multiplicity of 1 pfu/cell with either CV787 or CV802 (Ad5). Cell survival (viability) comparing the effect of the E3 region with CV739 and CV787 in LNCaP cells was assessed by measuring mitochondrial activity using MTT (17).

***In Vivo* Animal Experiments.** LNCaP xenograft tumors in *nu*/*nu* were induced in 6–7-week-old BALB/c *nu*/*nu* mice and allowed to grow to an average volume of 300 mm^3 (6). Fifty μl of CV787 in PBS-10% glycerol or PBS-10% glycerol alone were injected into the tail vein. Tumor size was measured weekly, as described previously (6). Serum samples were collected by tail vein incision. PSA levels were measured using an immunoassay kit (Genzyme Diagnostics, San Carlos, CA).

Results and Discussion

Specificity of CV787. The target cell specificity of CV787, relative to that of wt Ad5, was tested in Ad5 $E1A^+$ and $E1B^+$ 293 cells, PSA^+ human LNCaP prostate cells, and a panel of PSA^- cells, including human breast epithelial HBL-100 cells, ovarian cancer OVCAR-3 cells, and PA-1 cells. Fig. 2 shows the 48-h burst sizes of CN706, CV739, CV787, and CV802 (wt Ad5). The burst size of CV802 (wt Ad5) in these cells ranged from 3×10^3 to 2×10^4, and

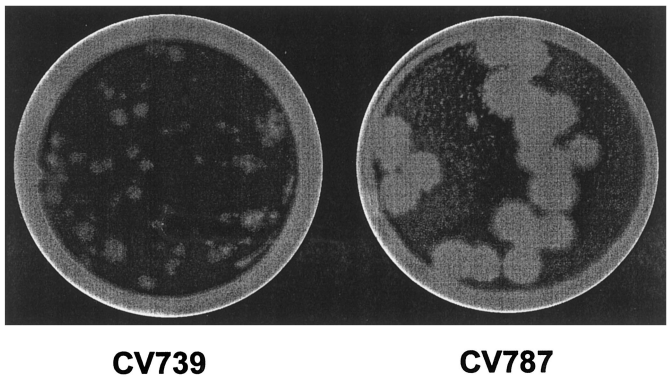

Fig. 4. Plaque morphology of CV739 and CV787. 293 cells were infected with CV739 and CV787. After a 4-h adsorption period, plates were overlayed with agar and incubated for 10 days. After 10 days, the agar overlay was removed, and the cells were stained with crystal violet.

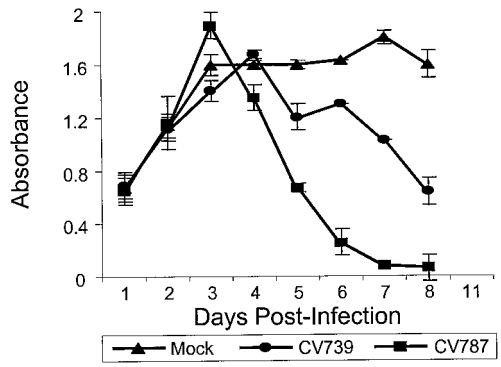

Fig. 5. Comparison of CV739 and CV787 on mitochondrial activity of infected LNCaP cells. Cells were infected with CV739 and CV787 at a multiplicity of infection of 1, and mitochondrial activity was measured by the MTT assay at the indicated time intervals.

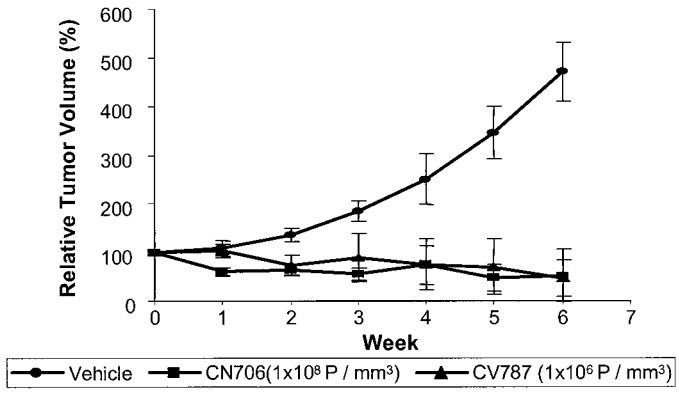

Fig. 6. Comparison of CN706 and CV787 i.t. injection activity toward LNCaP xenografts. *nu/nu* mice with s.c. LNCaP tumors (average size, 300 mm^3) were injected once into the tumor with 1×10^8 particles/mm^3 tumor of CN706 or 1×10^6 particles/mm^3 tumor of CV787.

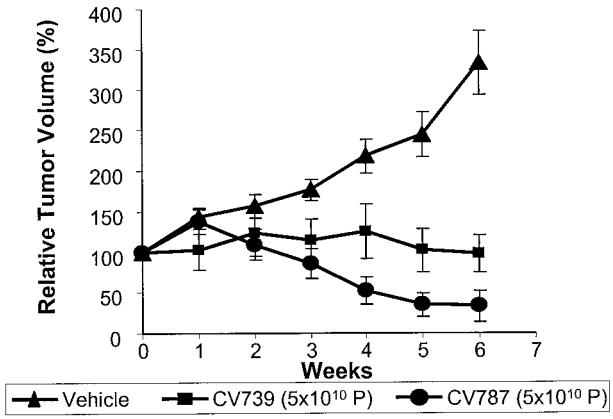

Fig. 7. Comparison of CV739 and CV787 i.v. injection activity toward LNCaP xenografts. *nu/nu* mice with s.c. LNCaP tumor xenografts (average size, 300 mm^3) were injected once into the tail vein with 5×10^{10} particles of CV739 or CV787.

the burst size of CN706 ranged from 3×10^3 to 1×10^4 in 293 cells and the PSA^+ cell line LNCaP; however, in all PSA^- cell lines, the burst size ranged from 1×10^1 to 8×10^1. The burst sizes of CV739 and CV787 were $\sim 10^4$ in 293 cells and the PSA^+ cell line LNCaP and were less than 1 to 1×10^{-2} in all PSA^- cell lines. Failure of CV787 to reach a burst size of 1 by 48 h following infection with a multiplicity of 2 indicates an essentially complete inability to replicate in cells lacking transcription factors that are capable of interacting with PSE. CV787 is capable of replicating but to an extremely limited extent in PSA^- cells, as shown by its growth curve in hMVEC cells (Fig. 3). In these cells, CV787 replicated 10–20-fold during 4 days following infection with a multiplicity of 2 pfu/cell, but in which the burst size, in agreement with the results presented above, failed to reach 1. In contrast, CV802 (wt Ad5) replicated 10^6-fold and reached a burst size of 10^4.

Specificity of CV787 was also shown by cell survival of PSA^- HBL-100 and OVCAR-3 cells, as measured by the MTT assay, compared with the destruction of PSA^+ LNCaP cells. Whereas CV802 destroyed all three cell types, CV787 only destroyed PSA^+ LNCaP cells (data not shown). Specific cell destruction was also measured in hMVEC monolayers infected for 10 days with multiplicities ranging from 0.01 to 10. Data similar to those previously published for CV764 (7) show that, even at an infecting multiplicity of 0.01, CV802 (wt Ad5) caused very substantial cytopathic effects; in contrast, CV787 caused minimal cytopathic effects in 10 days, even at an infecting multiplicity of 10 (data not shown). Together, these results confirm the conclusion that CV787 is almost totally attenuated for PSA^- cells but replicates normally in PSA^+ cells.

Increased Efficacy of CV787 Due to the Ad5 E3 region. The increased efficacy resulting from the incorporation of the Ad5 E3 region was first shown by *in vitro* tests. A representative plaque assay of CV739 ($E3^-$) and CV787 ($E3^+$) on 293 cells is shown in Fig. 4. Plaques of E3-deleted Ad5 are substantially smaller and less distinct than those of E3-containing adenovirus 10 days after infection. Plaques of CV787 appear more than 3-fold larger than plaques of CV739. $E3^-$ CN702 and $E3^-$ CN706 produced plaques similar to those shown for $E3^-$ CV739, whereas $E3^+$ CV802 produced plaques similar to those shown for $E3^+$ CV787 (data not shown). A similar picture of efficacy emerged when LNCaP cell survival was assessed by measuring mitochondrial activity (17). CV787 completely eliminated mitochondrial activity 7 days after infection, whereas CV739 cells still had 67% the mitochondrial activity of uninfected cells 7 days after infection (Fig. 5).

The increased efficacy of incorporation of the Ad5 E3 region was also shown *in vivo*, as measured in the LNCaP *nu/nu* mouse xenograft model. BALB/c *nu/nu* mice harboring s.c. 300-mm^3 LNCaP xenografts located on the back were injected either i.t. or i.v. into their tail veins with either vehicle (50 μl of PBS-10% glycerol) or the same volume of vehicle containing CN706, CV739, or CV787. Fig. 6 shows the result of a single i.t. treatment of LNCaP tumors with 1×10^8 particles/mm^3 tumor CN706 or 1×10^6 particles/mm^3 tumor CV787. Both viruses yielded the same degree of tumor reduction, implying a 100-fold increase in efficacy of CV787, compared with CN706. In addition, 1×10^8 particles/mm^3 tumor CV787 completely eliminated LNCaP tumors by a single i.t. injection (data not shown). Differential

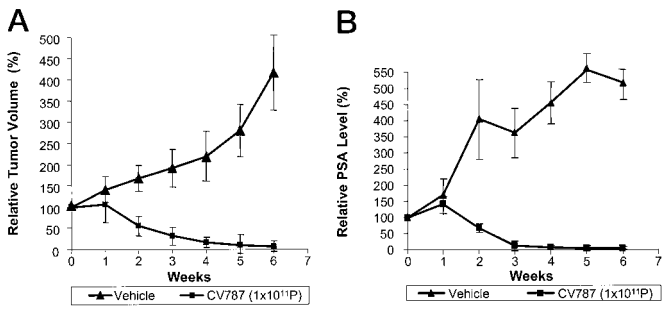

Fig. 8. LNCaP xenograft tumors in *nu/nu* mice following one i.v. tail vein injection of CV787. *A*, tumor size, measured weekly. *B*, serum PSA, measured weekly by immunoassay. *C*, replication of adenovirus in LNCaP tumors determined by immunohistochemistry with rabbit polycolonal antibody to Ad5.

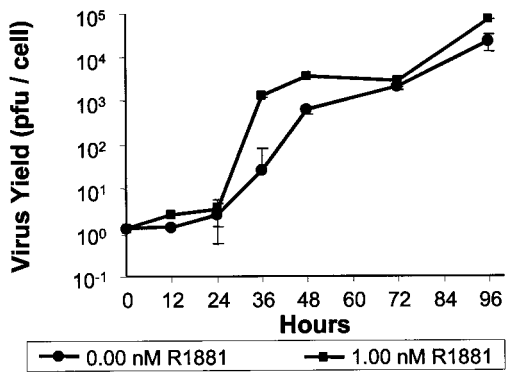

Fig. 9. Growth of CV787 in LNCaP cells with or without R1881. LNCaP cells were infected with CV787 at a multiplicity of infection of 2. Following adsorption, the cells were washed twice with PBS, and medium with or without R1881 was added. Duplicate cultures were harvested at the indicated time points and subjected to three cycles of freeze-thaw, and virus titers were determined in triplicate on 293 cells.

efficacy toward LNCaP tumors between CV739 and CV787 was also shown following a single i.v. administration of 5×10^{10} particles. Fig. 7 shows that CV739 could stop tumor growth at this dose level but that CV787 caused a 4-fold reduction in tumor volume.

i.v. Administration of CV787. Perhaps most importantly, CV787 could eliminate preexistent distantly located LNCaP tumors. Six weeks following a single i.v. injection of 1×10^{11} CV787 particles, the sizes of the tumors were reduced to less than 5% of their original size (Fig. 8*A*), and 8 of 14 mice were visually free of tumors. Those tumors that were still present were immunohistologically devoid of PSA (data not shown). The serum PSA levels in mice injected with buffer increased (Fig. 8*B*), whereas the levels in mice injected with CV787 decreased to ~5% of their starting values within 3 weeks. Virus replication within LNCaP xenografts could be shown at both 7 and 28 days after injection, as evidenced by Ad5 immunostaining (Fig. 8*C*). As controls, similar experiments were carried out with *nu/nu* mice carrying LoVo (colon cancer) or Hep3B (hepatoma) xenografts. These xenografts continued to grow normally following tail vein injection of CV787 (data not shown).

Finally, hormone-refractory patients continue hormone therapy, even if such hormone ablation therapy is no longer effective (2, 3). If CV787 is to be considered to treat end-stage metastatic hormone-refractory prostate cancer, it is important to know whether CV787 can grow in the absence of testosterone. Fig. 9 shows the one-step growth curve of CV787 in LNCaP cells in the presence of the stable but biologically active testosterone analogue R1881 at 1 nM and in the absence of R1881. CV787 grows normally in the absence of R1881 and achieved a burst size in excess of 10^4 pfu/cell.

In summary, CV787 is an ARCA that replicates like wt Ad5 in cells that express PSA but that is attenuated 10,000–100,000-fold with respect to replication in PSA^- cells. CV787 is highly cytopathogenic in PSA^+ cells and is capable of eliminating distantly located preexisting prostate tumors following i.v. injection. The addition of the Ad5 E3 region increases efficacy 10–100-fold both *in vitro* and *in vivo*. Furthermore, CV787 with the complete E3 region can be expected to resist many of the host antiviral MHC-1 defense mechanisms of wt Ad5 (15–17, 25). Finally, CV787 can replicate normally in the absence of testosterone and could, thereby, help patients continuing hormonal ablation therapy. CV787 is a potentially powerful therapeutic for human metastatic cancer.

Acknowledgments

We thank Young Kim, Heather Conners, and Andy Little for technical assistance. We also thank Dr. W.K. Joklik for suggestions in preparing and editing the manuscript.

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Cancer Res 1999;59:4200-4203.