Use of the Hollow Fiber Assay for the Discovery of Novel Anticancer Agents from Fungi

By: Cedric J. Pearce, Daniel D. Lantvit, Qi Shen, David Jarjoura, Xiaoli Zhang, Nicholas H. Oberlies, David J. Kroll, Mansukh C. Wani, Jimmy Orjala, Djaja D. Soejarto, Norman R. Farnsworth, Esperanza J. Carcache Blanco, James R. Fuchs, A. Douglas Kinghorn, and Steven M. Swanson

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Abstract:

The hollow fiber assay (HFA) is a drug discovery tool to aid investigators in the prioritization of lead compounds identified by in vitro testing for further development in animal models of disease. In the HFA, cells are cultured in hollow fibers containing pores of a diameter (500 kDa) large enough for proteins and other macromolecules to enter, but too small for the cells to escape. The fibers are filled with cells, sealed and placed in the peritoneal cavity of immunodeficient mice. The mice undergo a predetermined treatment regimen after which the fibers are retrieved and the cells evaluated for activity of a target relevant to the disease modeled. The HFA combines advantages of both in vitro and in vivo assay systems. It uses the same cell lines used in culture systems, is a rapid assay, and requires fewer animals and less test substance than conventional xenograft systems. Like traditional in vivo assays, the test substance is evaluated in a live animal, which affords an initial assessment of associated toxicity and pharmacokinetic properties of the test substance.

Keywords: Drug discovery | Fungi | Animal models | Natural products | Cancer

Article:

1. Introduction

While the hollow fiber assay (HFA) can be adapted for a variety of uses (1, 2, 3, 4), our focus has been on the discovery of novel anticancer agents derived from natural sources such as fungi (5). Hollingshead and colleagues originally developed the assay as a means of prioritizing the many lead compounds that had been identified in the 60-cell line panel at the National Cancer Institute (NCI) (6). When the Cancer Chemotherapy National Service Center was established at the NCI in 1955, the primary screening tool was transplantable murine cancer models, which involved a vast number of mice. While these models were critical for the discovery of many effective anticancer agents used clinically, the leadership at the NCI decided that using human cancer cell lines would provide a greater diversity of targets relevant to human cancers and thereby increase the chance of success in their drug discovery program. Researchers at the NCI collected and characterized a panel of 60 cell lines representing cancers of the lung, colon, brain, ovary, breast, prostate, and kidney, together with leukemia and melanoma. These cells are also capable of propagating in immunodeficient mice as xerographs allowing for leads identified in the cell line panel to be followed up in vivo. As high-throughput and automated procedures were optimized using in vitro assays, in vivo models became the bottleneck for moving compounds forward in the drug discovery process. Since the HFA can be completed in about 1 week and requires only three mice per dose for each test substance, it allows investigators to prioritize lead compounds efficiently by saving time and using fewer animals (4).

Fungi are recognized as one of the more significant sources of medicines and have provided a number of spectacular advances in the treatment of human diseases as is illustrated by the discovery of penicillin G, and the introduction of the first statins, for example. Even after decades of using fungal metabolites as leads for new drugs they remain a viable alternative to purely synthetic routes. For example, Gilenya™ (fingolimod, Novartis) is a compound based on myriocin, which is a metabolite first found in an insect-colonizing fungus, *Isaria sinclairii*. Gilenya™ was approved by the US Food and Drug Administration as the first orally active medicine for multiple sclerosis. These medicines represent the best of a very large catalogue of bioactive compounds produced by fungi. The chemical diversity of fungal metabolites is extraordinarily broad and this is reflected in their biological activity.

A number of fungal metabolites and their derivatives have been evaluated clinically as potential cancer medicines; examples include brefeldin A, wortmannin, cytochalasin E, and related derivatives such as fumagillin, gliotoxin, terrecyclic acid A, the illudin derivative irofulvin, and lentinan and related polysaccharides which are approved for use in Japan. In our own program to find new cancer lead compounds funded by the NIH (Discovery of Anticancer Agents of Diverse Natural Origin. 5P01 CA125066; Principal Investigator, A.D. Kinghorn supported by the NCI, National Institutes of Health), over the course of the past 4 years we have discovered 102 compounds, 31 of which are novel, that are active in our cultured cancer cell lines. Our other programs using vascular plants and cyanobacteria have also been successful in finding new compounds. This success means that we are in urgent need of secondary bioassays to help us focus on those leads with the highest probability of generating clinical lead-quality compounds, and the need for methods to evaluate compounds in vivo is especially useful. To this end we have been developing methods to provide such prioritization, and the HFA has been one approach we have taken.

2. Materials

2.1 Cell Culture Supplies

1. Human cancer cell lines (American Type Culture Collection; *see* Note 1).

2. RPMI 1640 medium with glutamine.

3. Fetal bovine serum.

4. Trypsin (0.05%)/EDTA.

5. Antibiotic/antimitotic, 100× [contains penicillin (10^4 units/ml), streptomycin (10^4µg/ml), and the antifungal agent amphotericin B (25 µg/ml)].

6. Trypan blue solution (0.4%, Sigma Chemical Co.).

7. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].

8. Tissue culture flasks (T75 and T150, BD Falcon).

9. Sterile centrifuge tubes (15 and 50 ml, BD Falcon).

10. Hemocytometer (Reichert Bright-Line, Hausser Scientific, Horsham, PA).

2.2 Hollow Fiber Preparation

1. Modified polyvinylidene difluoride hollow fibers (Spectrum Labs, CellMax®, molecular weight cutoff of 500 kDa).

2. Dry glass bead sterilizer (*see* Note 2).

3. Needle holders, forceps, and scissors.

4. 1 and 5 ml syringes; needles.

5. Stainless steel pan (approximately 10 × 6.5 in.).

6. Six-well culture plates.

7. Medical gloves (latex or nitrile).

2.3 Surgical Supplies

1. Trocar (11 gauge; Popper and Sons, New Hyde Park, NY).

2. Wound clip system (Autoclip®, Harvard Apparatus).

3. General operating scissors.

4. Dressing and tissue forceps.

3. Methods

3.1 Conditioning Fibers

Wear gloves when handling fibers.

1. Using a syringe fitted with either an 18 or 20 gauge needle, flush each fiber individually with 70% EtOH (Fig. 1).

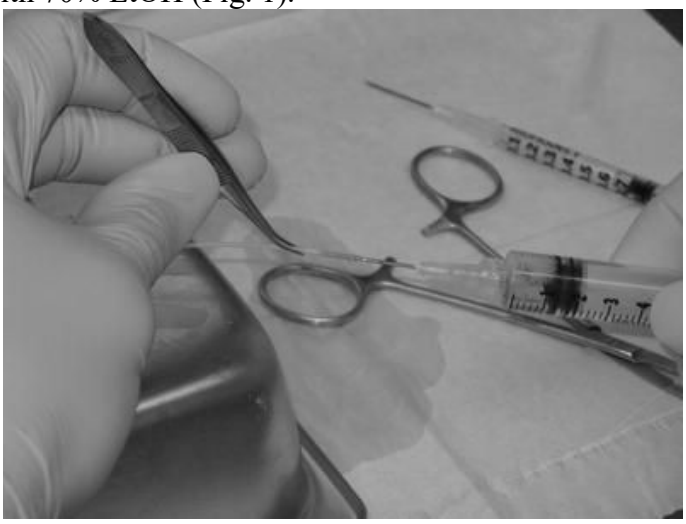

Figure 1. Sterilize the fibers. Flush the fibers with 70% ethanol using a syringe fitted with an 18 or 20 gauge needle.

2. Place fibers in an autoclavable covered pipette tray filled with 70% EtOH for at least 72 h. Do not allow fibers to dry out from this point on.

3. After 72 h, use a needle and syringe as above and flush fibers twice individually with deionized water.

4. Place fibers in another pipette tray filled with deionized water and autoclave for 30 min. Let cool and store at 4°C until fibers are needed.

3.2 Cell Culture

The reader is referred to books on cell culture for general procedures (7, 8). The cells are grown in RPMI 1640 medium supplemented with glutamine and 10% fetal bovine serum in T75 flasks (8). The split ratio is dependent on the rate of proliferation, which varies from line to line, but generally is between 1:3 and 1:10. Cells are split at a ratio that will result in 70–80% confluence on the experiment day. On the day before the experiment, cells are fed by removing half of the medium and adding an equal volume of fresh medium. On the day that the fibers are to be filled,

the cells are washed with PBS, incubated with 1–3 ml of trypsin, and incubated for 5–10 min at 37°C to detach them from the flask surface. The cells are resuspended in medium, counted using a hemocytometer, and plated in new flasks or injected into preconditioned hollow fibers.

1. Pour off medium from cells into a sterile tube and place on ice (this conditioned medium will be used to resuspend the cells later); wash cells twice with PBS.

2. Aspirate PBS and add 2 ml trypsin per flask. Incubate at 37°C for 5 min to detach the cells from the flasks.

3. Add 10 ml fresh medium to flask; transfer medium and cells to a sterile centrifuge tube and centrifuge (5 min, 800 × *g*, 4°C); aspirate medium, resuspend cells in 5 ml conditioned medium, and place on ice.

4. Determine viable cell number using the trypan blue dye exclusion test (9).

5. Add the appropriate volume RPMI 1640 with FBS medium (20% vol/vol) to achieve the desired cell concentration and place on ice.

3.3 Fiber Preparation

Conditioning, filling, and sealing the hollow fibers must be conducted in a sterile environment.

1. Mark the bottom of a stainless steel pan every 2 cm for work surface. Wrap the stainless steel pan in aluminum foil. Autoclave needle holder with scissors, smooth forceps, and stainless steel work surface (*see* Note 3).

2. Empty a biosafety cabinet and wipe down with 70% EtOH. Place pipette tray containing fibers and bead sterilizer in the biosafety cabinet. Spray down the outside of the tray with EtOH. Allow bead sterilizer to heat to maximum temperature.

3. Place centrifuge tubes of cells and media on ice. Set up a sterile field in the cabinet by placing sterile drapes on the work surface. Carefully unwrap the stainless steel work surface under the hood and place in the sterile field. Place all autoclaved instruments in the sterile field. Remove 10 and 1 ml syringes and 18 or 20 gauge needles from freezer and place in the sterile field.

4. Don sterile gloves. Uncap cold media tube under hood and hold while 10 ml syringe is filled. Once syringe is filled, tube is removed from hood. Cold media is dispensed on the work surface to keep fibers from drying out during the filling process. Syringe with the remaining media is placed in the sterile field.

5. Carefully mix cell suspension to create homogeneity. Under the hood, uncap the cold cell suspension tube and fill a 1 ml syringe; place it in a sterile field within the hood. Remove lid from pipette tray and, with sterile forceps, remove 2–3 fibers and place on

medium-soaked work surface. Flush each fiber intraperitoneally with cold medium to remove water.

6. Create an air bubble in the 1 ml syringe containing the cell suspension so that the remaining medium can be pushed out of the fiber before filling it with the cell suspension. Insert needle into one end of the fiber. With the needle pointing upwards, form an upside-down "U" with the fiber before dispensing air bubble and cell suspension (Fig. 2). Fill fiber with cold cell suspension avoiding air bubbles in the fiber (*see* Note 4).

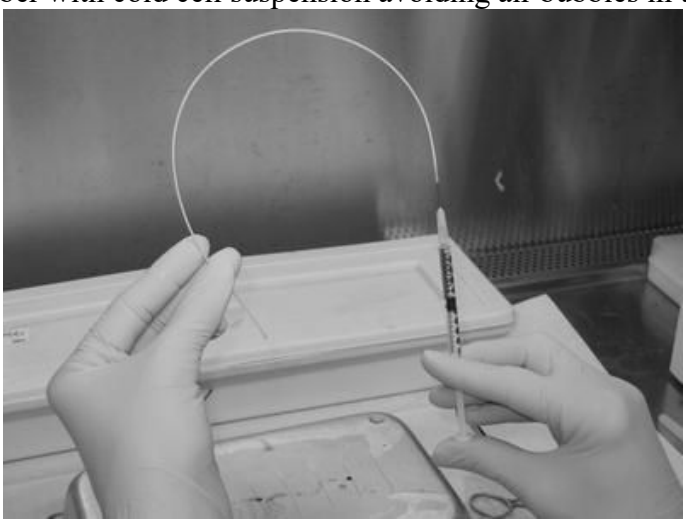

Figure 2. Filling the fibers with cells. Medium containing cells is gently injected into the sterile fiber behind a cushion of air, replacing the medium lacking cells.

7. Once the total length of the fiber is filled with the cell suspension, create a "d" by taking the loose end of the fiber and moving it horizontally to the work surface (Fig. 3). Heat the needle holder in the bead sterilizer for a few seconds and heat-seal the open end by clamping down on the fiber with the needle holder. Then heat-seal the other end just below the needle. Lay the filled fiber on the work surface. Continue until each of the 2–3 fibers is filled. Be sure to keep fibers from drying out by keeping them covered with cold media.

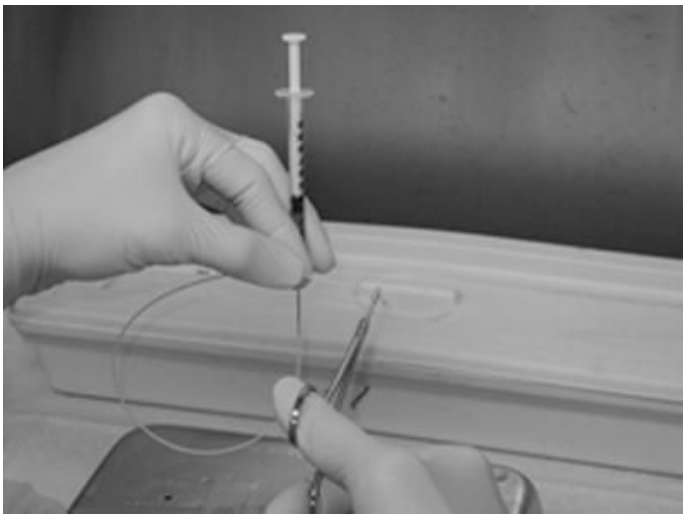

Figure 3. The ends of the fibers containing cells are sealed by pinching with a heated hemostat.

8. One at a time, the filled fibers are laid across the area of the work surface that has the 2 cm demarcations. Again heating the needle holder a few seconds, a mark is made every 2 cm for the length of the fiber by pressing the needle holder closed on the fiber (Fig. 4). Repeating the process above a second time at each mark this time applying a slightly pulling action on the fiber creates a clear area that completes the heat-seal. Cut in the center of the heat-seal to make individual fibers for implantation.

Figure 4. Cutting the individual fibers for implantation. Using a heated hemostat, clamp down firmly on the cell-filled fibers at 2 cm intervals. Using a scalpel or surgical scissors, cut the fibers in the middle of the heat-seal to generate the individual fibers for implantation.

9. Fibers are placed into each well of a 6-well culture plate filled with 2 ml of medium supplemented with serum but lacking antibiotics. Place fibers in a 37 °C incubator (*see* Note 5).

3.4 Implantation of Fibers

1. Remove 6-well plates containing fibers from the incubator and check for sterility. Three fibers from each cell line are set aside to serve as “day 0” controls (see below).

2. Anesthetize female athymic mice NCr nu/nu mice at 5–6 weeks of age with a combination of ketamine and xylazine (*see* Note 6).

3. Lay mouse on its side and make a small incision through the skin and muscle layers just below the spleen. The size of the incision should be just large enough to accommodate the diameter of the fibers. Carefully handle fibers with forceps avoiding excess pressure and place the appropriate fibers in the abdominal cavity. Suture the body wall incision and use a wound clip to close the skin incision.

4. Lay the same mouse on its stomach and make a small incision at the nape of the neck. Fill trocar with appropriate fibers, insert trocar into the incision, and place fibers over the

hip region as you begin to withdraw trocar. Repeat until the appropriate number of fibers is placed in the animal. Close incision with a wound clip.

5. Place animals back in cage and allow them to recover. Intraperitoneal injections of test compounds begin on day 3 after implantation and are usually injected daily for 4 days. Animal body weights are recorded every day to monitor compound toxicity (*see* Note 7).

6. On day 7, animals are sacrificed and fibers are retrieved and placed in 6-well plates filled with warm media. Wells are labeled according to treatment and site of implantation. Fibers are placed in the incubator for at least 30 min to allow cells to normalize.

7. The viable cell mass is evaluated by the MTT assay described below. The percent net growth for each cell line in each treatment group is calculated by subtracting the day-zero absorbance from the day 7 absorbance and dividing this difference by the net growth in the day 7 vehicle-treated controls minus the day-0 values. A 50% or greater reduction in net cell growth in the treated samples compared to the vehicle control samples is considered a positive result (*see* Note 8).

3.5 MTT Assay

We have modified this commonly used assay, which measures the reduction by metabolically active cells of a yellow tetrazolium salt to a purple formazan. The procedure has been modified for the HFA from published standard protocols (10, 11).

1. Prepare stock solution of 5 mg/ml MTT. Store at 4 °C and protect from light.

2. Prepare a 2.5% solution of protamine sulfate in saline and sterilize by passing through a 0.2 μm filter; store at 4 °C.

3. Prepare a 1 mg/ml working solution of MTT by adding 4 ml of media to 1 ml of stock solution. Warm in a 37 °C water bath.

4. Add 1 ml of working solution to the 2 ml of media for each well containing harvested fibers. Incubate for 4 h in cell culture incubator.

5. Aspirate all liquid from each well. Wash fibers by adding 2 ml of 2.5% protamine sulfate to each well containing fibers. Store at 4 °C overnight.

6. Aspirate liquid and perform a second wash with 2.5% protamine sulfate. Incubate at 4 °C for at least 2 h. Remove fibers and gently dry with a KimWipe. Cut fiber in half and place one fiber per well of a 24-well plate making sure that each well is labeled with correct treatment and placement.

7. Allow the fibers to dry overnight protected from light (*see* Note 9).

8. Add 250 µl of DMSO to each well and cover plate with foil. Place plates on a shaker for 4 h.

9. Remove 150 µl of solution from each well and place in a 96-well plate. Read absorption at 490 nm on a microplate reader.

4. Notes

1. The cell lines are selected from the list of 30 lines used in the HFA developed by Hollingshead et al. (12). Since we are looking for new proteasome inhibitors, we have chosen lines that are most sensitive to bortezomib, an FDA-approved proteasome inhibitor. This information was obtained by querying the database of therapeutics for their activity against the NCI's 60 cell line panel (13).

2. Several methods are acceptable for sterilizing the hemostat used to seal the fibers. We use a glass bead sterilizer because it can rapidly heat and sterilize metal instruments such as the hemostat that is used to heat-seal the cell-filled fibers.

3. Each cell line requires its own set of equipment to avoid cross-contamination.

4. Each cell line should be represented by a different color fiber. If a fiber dries out in the process (turns white), it is unusable.

5. Incubation allows cells to attach to the fiber surface prior to implantation in the mice. Six fibers are not implanted in the mice, but remain in the incubator and are harvested when the mice are sacrificed to assure that they are free of microbial contamination.

6. Although most laboratories use immunodeficient mice, Shnyder et al. (14) have reported that immunocompetent mice such as NMRI also can be used at significantly lower cost. Ketamine and xylazine are administered by intraperitoneal injection at 100 and 10 mg/kg, respectively.

7. The dose levels chosen for each test compound are determined by performing acute toxicity tests for each agent as described by the NCI Developmental Therapeutics Program (15). One mouse is given a single ip injection at 400 mg/kg body weight; another mouse is administered 300 mg/kg and a third mouse is given 100 mg/kg. The mice are observed for 2 weeks and sacrificed if they lose 20% or more of their body weight or exhibit outward signs of toxicity as indicated above. If all three mice die or must be sacrificed, three lower doses (e.g., 50, 25, and 10 mg/kg) are tested. The process is repeated until the maximum tolerated dose (MTD) is identified. We routinely use 40% of the single-dose MTD as the highest dose in our four-daily-dose treatment schedules. The initial level of exposure that we choose for the acute toxicity study is based on the activity of the compound in cell cytotoxicity tests.

8. In the HFA, the measure of efficacy is the percentage reduction in cells at day 6 as compared to day 0 (implantation date):

$$\%\text{netreduction} = \frac{(\text{day0OD}_{540} - \text{day6OD}_{540})}{\text{day0OD}_{540} \times 100\%},$$

where OD_{540} is the optical density measure of cell numbers. Because the maximum reduction is 100%, nonlinear regression modeling of dose effects should be used. Models would be estimated separately for implant site (subcutaneous vs. intraperitoneal), and for each cell line. The maximum dose can be determined from prior toxicity studies. With multiple estimated models per compound, some approach to decision making about further experimentation (e.g., xenograft testing) with the compound must be chosen. Depending on circumstances, an investigator can choose to focus on minimizing false negatives (possibly missing promising compounds) or false positives (expending further resources on ineffective compounds).

Decker et al. (12) developed a scoring system that seems to emphasize avoidance of false positives. Based on their recommendation, promising compounds are those that are effective across multiple cell lines. They validate their scoring system through the prediction of success in multiple xenograft models. A score is considered low if a compound acts only on one cell line. They also recommend further testing of any compound that produces 100% cell death. Therefore if one were most concerned about false negatives, such a scoring system would not be optimal. Instead, one could base the decision to move forward with a compound on the percent net reduction observed at the maximum dose for a particular cell line. A confidence interval on the percent net reduction estimate would be useful to take into consideration sampling error of the estimate. A more formal decision procedure could test the significance of the dose–response curve, and use liberal criteria for declaring a positive result. The number of cell lines and compounds tested simultaneously must be considered when deciding on such criteria. This approach further allows for a second more precise study when results are too variable to achieve significance.

9. We place the plates containing the fibers near the vent of a biological safety cabinet with some foil loosely applied to protect the dye from light.

References

1. Hassan SB, de la Torre M, Nygren P, Karlsson MO, Larsson R, Jonsson E (2001) A hollow fiber model for *in vitro* studies of cytotoxic compounds: activity of the cyanoguanidine CHS 828. Anticancer Drugs 12:33–42

2. Hollingshead M, Roberson J, Decker W, Buckheit R Jr, Elder C, Malspeis L, Mayo J, Grever M (1995) *In vivo* drug screening applications of HIV-infected cells cultivated within hollow fibers in two physiologic compartments of mice. Antiviral Res 28:265–279

3. Hollingshead MG, Bonomi CA, Borgel SD, Carter JP, Shoemaker R, Melillo G, Sausville EA (2004) A potential role for imaging technology in anticancer efficacy evaluations. Eur J Cancer 40:890–898

4. Mi Q, Pezzuto JM, Farnsworth NR, Wani MC, Kinghorn AD, Swanson SM (2009) Use of the *in vivo* hollow fiber assay in natural products anticancer drug discovery. J Nat Prod 72:573–580

5. Kinghorn AD, Chin YW, Swanson SM (2009) Discovery of natural product anticancer agents from biodiverse organisms. Curr Opin Drug Discov Devel 12:189–196

6. Hollingshead MG, Alley MC, Camalier RF, Abbott BJ, Mayo JG, Malspeis L, Grever MR (1995) *In vivo* cultivation of tumor cells in hollow fibers. Life Sci 57:131–141

7. Davis J (2011) Animal cell culture: essential methods. Wiley, Hoboken

8. Freshney RI (2010) Culture of animal cells: a manual of basic technique and specialized applications, 6th edn. Wiley-Liss, Hoboken

9. Strober W (1997) Trypan blue exclusion test of cell viability. Curr Protoc Immunol, A.3B.1–A.3B.2

10. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

11. Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A, Boyd MR (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Res 51:2515–2520

12. Decker S, Hollingshead M, Bonomi CA, Carter JP, Sausville EA (2004) The hollow fibre model in cancer drug screening: the NCI experience. Eur J Cancer 40:821–826

13. Developmental Therapeutics Program, US National Cancer Institute. http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp. Accessed July 28, 2012.

14. Shnyder SD, Hasan J, Cooper PA, Pilarinou E, Jubb E, Jayson GC, Bibby MC (2005) Development of a modified hollow fibre assay for studying agents targeting the tumour neovasculature. Anticancer Res 25:1889–1894

15. Developmental Therapeutics Program, US National Cancer Institute. http://dtp.nci.nih.gov/branches/btb/acute_tox.html. Accessed July 28, 2012.