This undertaking has, however, proven challenging because there are 12 catalytically active human CA isoforms, which are structurally homologous and share high sequence identity [26, 43]. Additionally, most of the conserved residues are located within the active site [26]. At the core of the human CA active site, a zinc metal is tetrahedrally coordinated to three imidazole rings from His 94, 96, and 119 (using CA II numbering) [44–46]. This coordinated zinc metal is essential for the catalytic conversion of carbon dioxide into bicarbonate and a proton in the presence of water, the physiological reaction catalyzed by CAs [25, 44]. The catalytic site is located in an ~15 Å conical cavity and is surrounded by both hydrophobic and hydrophilic regions that provide accessibility to solvent, carbon dioxide, bicarbonate, and inhibitors [26, 47].

Recently, a "selective pocket" was proposed to extend between 10 and 20 Å away from the catalytic zinc that may serve to aid in the design of CA isoform selective inhibitors [47]. This pocket, containing several amino acids that differ among the CA isoforms, is currently the focus for designing isoform specific inhibitors, using the extended tail approach [26]. Other approaches have also been adopted in the development of isoform specific inhibitors that interact in the less conserved regions, outside of the active site, to inhibit catalysis [25]. CAs also exhibit a slower esterase activity mediated by the same catalytic pocket with a mechanism similar to that of the hydratase/CA activity [48]. Although the role of this second activity in cancer is currently unknown, many investigators often use the esterase reaction as an indicator of hydratase activity [49].

A variety of compounds and biologics have been described in the literature that target different CA isoforms in many disease models. The potency, specificity, and efficacy of these inhibitors have been tested using different techniques and approaches. In this review, we focus on some of the biophysical, biochemical, and cell based approaches currently studied to evaluate the potency of CA targeted inhibitors and to decipher the role of CAs in cancer. Some of the approaches discussed include those that investigate inhibition of the CA enzymatic activity like esterase activity assays and stop flow kinetics and biophysical methods such as thermal shift assays, surface plasmon resonance, mass inlet mass spectroscopy (MIMS), X-ray crystallography, Cryo-EM, neutron crystallography, and nuclear magnetic resonance (NMR) (Figure 1). Other approaches that will be discussed include large-scale gene and protein expression, measure metabolism, pH regulation, cell growth, migration, and invasion (Figure 2). Together, these methods will hopefully reveal the role of CAs in tumorigenesis, cancer progression, and metastasis.

2. Enzymatic Assays and Biophysical Methods That Evaluate the Potency of CA Inhibitors

There are many techniques and strategies that have been described in the literature that evaluate CA activity for both purified versions of the enzyme and "intact" cells. These studies have been performed in the presence and/or absence of CA targeting inhibitors. Some of the techniques include measuring CA esterase activity on an ester substrate or the conversion of carbon dioxide into bicarbonate and a proton, which include stop flow kinetics and MIMS. Biophysical approaches such as differential scanning fluorimetry (DSF), differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR) also demonstrate binding of CA inhibitors to different isoforms. These techniques, however, do not indicate the regions of the proteins where inhibitors bind or their mode of inhibition (Figure 1).

Structural biology techniques such as those discussed in this review, X-ray crystallography, Cryo-EM, neutron crystallography, and NMR, have been used to determine CA structures, most often in complex with inhibitors (Figure 1). These strategies have provided information related to the structure of several CA isoforms and the interactions with CA targeted inhibitors. Supuran and coauthors have developed several CA inhibitors, and their modes of binding have been determined using one or more of the abovementioned techniques. These inhibitors can be subdivided into five groups: (1) compounds that interact directly with the catalytic zinc (sulfonamides and their isosteres, dithiocarbamates and their isosteres, hydroxamates, etc.); (2) compounds that anchor to the zinc bound water/hydroxide ion (phenols, polyamines, sulfocoumarins, etc.); (3) compounds that occlude the CA active site entrance (coumarins and their isosteres); (4) compounds that bind out of the active site region (carboxylic acid derivatives); and (5) compounds with unknown binding mechanisms (secondary/tertiary sulfonamides, etc.) [25].

3. Enzymatic Assays (Figure 1(a))

3.1. Esterase Activity Assay. The CA esterase activity, which is similar in mechanism to its hydratase activity, was first observed and measured in the mid-1900s [48]. In this study, the authors showed that the esterase activity of human isoforms is significantly lower than that observed for the hydratase activity [48]. However, like the CA hydratase activity, the CA targeted small molecule inhibitor, acetazolamide, also inhibits its esterase activity [48]. Since the discovery of this similarity in inhibition, numerous studies have relied on the measure of CA esterase activity as a surrogate for selecting CA inhibitors that block hydratase activity [49, 50]. In this regard, a commercially available, high-throughput colorimetric assay has been developed to determine CA activity in biological samples, serum, and purified CA (BioVision Incorporated). This assay utilizes the esterase activity of an active CA on an ester substrate, which releases a chromophore. The released product (4-nitrophenol) can be readily measured and quantified using a microplate reader (absorbance 405 nm). In the presence of a CA specific inhibitor, the enzyme's decrease in activity directly correlates to the decrease in absorbance. Currently, most of the CA related work using this technique has focused on purified recombinant proteins assaying only one CA at a time. Perhaps this assay can be optimized to measure CA activity in either cancer cells or lysates from different samples (both tumor and normal) although this might prove challenging as biological samples are much more complex often with more than one CA present. Assigning the activity